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CORRIGENDA

Maleic anhydride in the study of naturally occurring isomers of vitamin A

By P. A. Plack

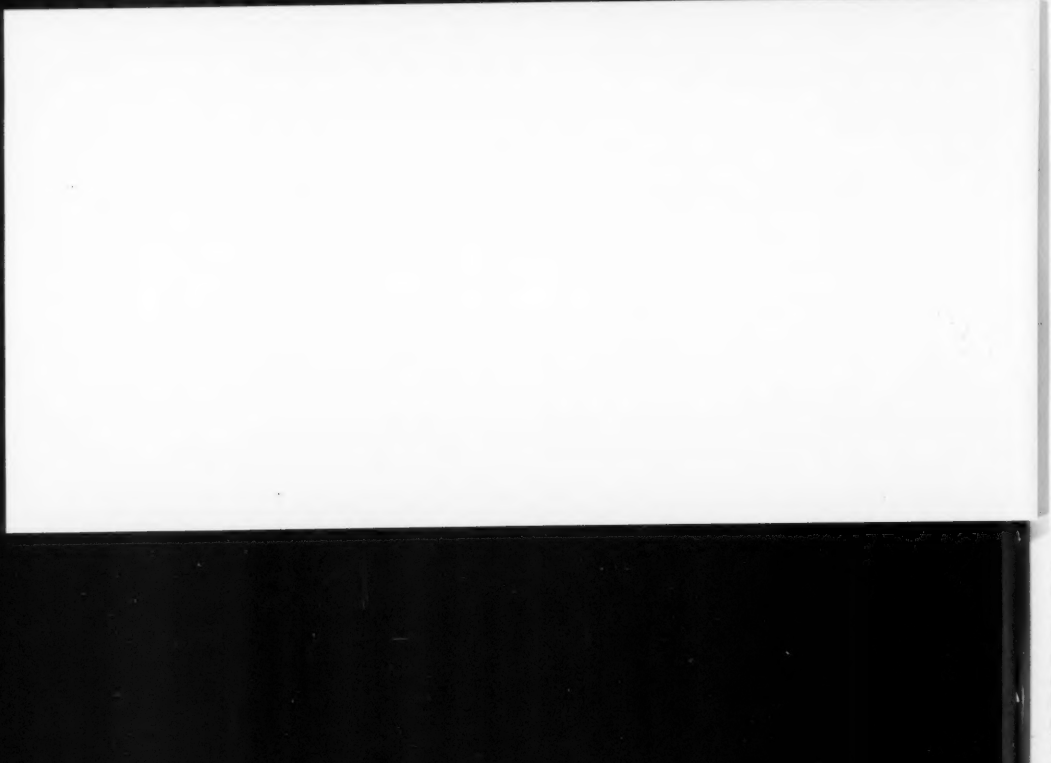
Volume 64 (1956)

p. 56, col. 2, lines 4–5:

for **Robeson *et al.* 1955*b*** ***read*** **Ames, Swanson & Harris, 1955**

p. 58, col. 2, line 21:

delete **(w/v)**



Some Observations on the Oxidative Degradation of Pteroyl-L-glutamic Acid

By J. A. BLAIR
University College of the Gold Coast

(*Received* 14 *May* 1956)

Several workers have studied the action of xanthine oxidase on pteroyl-L-glutamic acid (PGA). Jacobson & Good (1952) claimed that xanthine oxidase oxidized PGA and that the product had increased haemopoietic properties. Norris & Majnarich (1949) reported that incubation of xanthine oxidase with PGA gave material of increased haemopoietic power. On investigating a claim that PGA inhibited the oxidation of xanthine by xanthine oxidase, Kalckar & Klenow (1948) and Kalckar, Kjeldgaard & Klenow (1948, 1950) found that this inhibition was due to traces of 2-amino-4-hydroxypteridine-6-aldehyde usually present in PGA, and that this effect could be eliminated by incubating the PGA with xanthine oxidase. This was shown to be due to the oxidation of the aldehyde to 2-amino-4-hydroxypteridine-6-carboxylic acid by the enzyme. They found no change in the growth-promoting properties of PGA before and after incubation with xanthine oxidase. Rauen & Waldman (1950), Rauen, Waldman & Buchka (1951) and Yamamoto (1954) found that on incubating pig-liver homogenate with PGA, a bright, pale-blue fluorescent compound was formed. This was not identified.

It seemed desirable to repeat this work on the oxidation of PGA, with milk xanthine oxidase at stage M 5 as prepared by Avis, Bergel & Bray (1955). It has been confirmed that xanthine oxidase has no action on PGA; but when xanthine oxidase, methylene blue and PGA are incubated at 36–37° at a pH near neutrality, PGA is oxidized to 2-amino-4-hydroxypteridine-6-carboxylic acid. This oxidation proceeds by the intermediate formation of 2-amino-4-hydroxypteridine-6-aldehyde, which is then oxidized by the xanthine oxidase to 2-amino-4-hydroxypteridine-6-carboxylic acid (Lowry, Bessel & Crawford, 1949; Klenow, 1951). This is shown by the isolation of the aldehyde as the major product of the incubation of PGA with methylene blue.

MATERIALS

Pteroyl-L-glutamic acid. This was obtained commercially. By paper chromatography, with butanol–acetic acid–water (4:1:5, by vol.) as solvent, it was shown to be contaminated with 2-amino-4-hydroxypteridine-6-aldehyde and a trace of 2-amino-4-hydroxypteridine-6-carboxylic acid. The quantitative estimation of the aldehyde was made by the spot-area technique of Fisher, Parsons & Morrison (1948) and Fisher, Parsons & Holmes (1949). A portion (10 μl.) of a standard solution of the aldehyde (0·116 mg. in 1 ml. of 0·5 N-NH_3 soln.) and 10 μl. of a solution of PGA (3·73 mg. in 1 ml. of 0·5 N-NH_3 soln.) were placed on no. 1 Whatman paper, air-dried and developed with butanol–acetic acid–water (4:1:5, by vol.; ascending). After air-drying, the paper was viewed in ultraviolet light (365 mμ.) and the blue fluorescent spots of aldehyde were cut out and weighed. The amount of aldehyde present was estimated to be about 3% of the weight of PGA.

Xanthine oxidase. This was provided by the Chester Beatty Research Institute at stage M 5 of purification (Avis *et al.* 1955), in a solution of M phosphate, pH 6, containing 4 mg. of xanthine oxidase/ml. It was used without further purification.

2-Amino-4-hydroxypteridine-6-aldehyde. This was prepared by the method of Waller *et al.* (1950) with the modification that it was found necessary to irradiate the reacting solution with light from a 100 W lamp.

EXPERIMENTAL AND RESULTS

Incubation of PGA with xanthine oxidase

As PGA is readily decomposed by light, this and all other incubations were performed in darkness. Blank incubations were done where necessary to eliminate any possible side effects.

PGA (0·3 ml. of a solution of 0·3 mg./ml. in 0·15 M phosphate buffer, pH 7·7) and xanthine oxidase solution (0·1 ml.) were heated at 36–37° under aerobic conditions for 18 hr.

Paper chromatography of 10 μl. of the digest with butanol–acetic acid–water (4:1:5, by vol.) as solvent showed a faint pale-blue spot with R_F value close to that of 2-amino-4-hydroxypteridine-6-carboxylic acid. As judged by the size of the spot formed on the paper, the amount of acid formed did not appreciably increase after the first hour of incubation and represented less than 10% of the theoretical yield of carboxylic acid from PGA.

Incubation of PGA with xanthine oxidase and methylene blue

A solution (3 ml.) of 0·15 M phosphate buffer, pH 7·7, containing 1·56 mg. of PGA and 0·54 mg. of methylene blue was mixed with xanthine oxidase solution (1 ml.) and incubated at 36–37° under aerobic conditions for 18 hr.

Paper chromatography of 10 μl. of this digest on no. 1 Whatman paper with butanol–acetic acid–water (4:1:5, by vol.) showed on examination in ultraviolet light a large, bright, pale-blue spot with an R_F value close to that of 2-amino-4-hydroxypteridine-6-carboxylic acid, and a small, faint-blue spot corresponding to that of 2-amino-4-hydroxypteridine-6-aldehyde. The protein present in the digest seriously interfered with the chromatographic behaviour of the 2-amino-4-hydroxypteridine-6-carboxylic acid in other solvents. The bright-blue fluorescent substance was purified by chromatography on a column of cellulose powder (Whatman standard grade, column 1 in. diam., 14½ in. high) with 5 % (by vol.) aqueous acetic acid. The eluate containing the bright-blue fluorescent substance was evaporated to dryness and the residue taken up in 1 ml. of 0·5N-NH_3 soln. Determination of the ultraviolet spectrum of this fluorescent substance in 0·1N-NaOH showed two maxima, at 261 and 361 mμ. 2-Amino-4-hydroxypteridine-6-carboxylic acid has two maxima, at 262 and 365 mμ. (Mowat *et al.* 1948). The identity of the bright-blue fluorescent substance as 2-amino-4-hydroxypteridine-6-carboxylic acid was confirmed by comparison of its chromatographic behaviour with that of an authentic sample of the acid (Table 1).

Table 1. *Identification of oxidation products of PGA by means of paper chromatography*

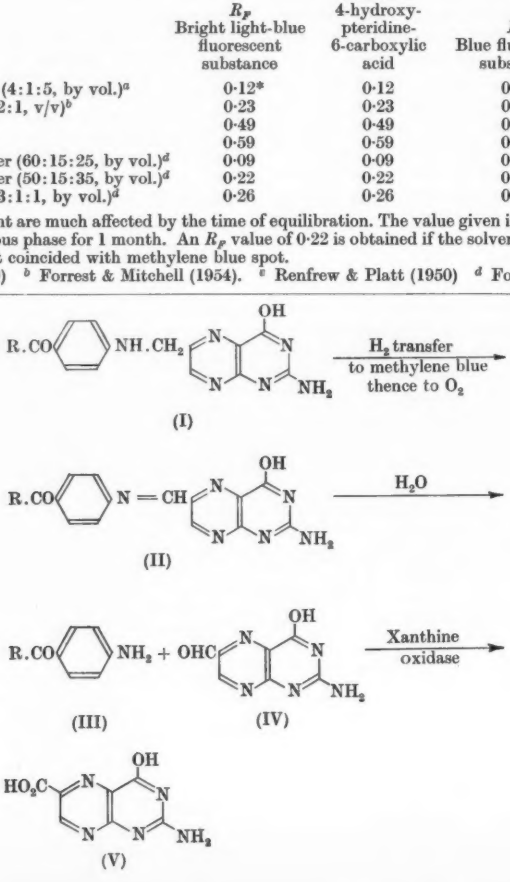
A volume (5 μl.) of the solution of the unknown and of the standard were run on the same chromatogram. Temp. 25–28°. Spots were viewed in ultraviolet light (365 mμ.).

	R_F Bright light-blue fluorescent substance	R_F 2-Amino-4-hydroxy-pteridine-6-carboxylic acid	R_F Blue fluorescent substance	R_F 2-Amino-4-hydroxy-pteridine-6-aldehyde
Butanol–acetic acid–water (4:1:5, by vol.)[a]	0·12*	0·12	0·33	0·33
Propanol–5 % acetic acid (2:1, v/v)[b]	0·23	0·23	0·46	0·46
5 % acetic acid[b]	0·49	0·49	0·52	0·52
3 % ammonium chloride[c]	0·59	0·59	0·43	0·43
tert.-Butanol–pyridine–water (60:15:25, by vol.)[d]	0·09	0·09	0·27†	0·28†
tert.-Butanol–pyridine–water (50:15:35, by vol.)[d]	0·22	0·22	0·54	0·54
Pyridine–propanol–water (3:1:1, by vol.)[d]	0·26	0·26	0·55	0·55

* R_F values in this solvent are much affected by the time of equilibration. The value given is for solvent which has been equilibrated with the aqueous phase for 1 month. An R_F value of 0·22 is obtained if the solvent is equilibrated for 3 days.

† Blue spot in the digest coincided with methylene blue spot.

[a] Good & Johnson (1949) [b] Forrest & Mitchell (1954). [c] Renfrew & Platt (1950) [d] Forrest & Todd (1950).



Quantitative estimation of the acid was carried out as follows. A portion (10 μl.) of the digest and 10 μl. of a solution of 2-amino-4-hydroxypteridine-6-carboxylic acid (0·1 mg./ml. of 0·5N-NH_3 soln.) were placed on Whatman no. 1 paper, air-dried and developed with butanol–acetic acid–water (4:1:5, by vol.; ascending). The paper was air-dried, viewed in ultraviolet light and the bright, pale-blue spots were cut out and weighed (Fisher *et al.* 1948, 1949). The yield of acid was about 60% of the theoretical yield from PGA.

Incubation of methylene blue and PGA

A solution (4 ml.) of 0·15M phosphate buffer, pH 7·4, containing 1·84 mg. of PGA and 1·04 mg. of methylene blue, was incubated at 36–37° under aerobic conditions for 18 hr. Paper chromatographic analysis of 10 μl. of the digest with butanol–acetic acid–water (4:1:5, by vol.) showed only a small spot corresponding to 2-amino-4-hydroxypteridine-6-carboxylic acid (R_F value 0·12), but a large blue fluorescent spot of R_F 0·33. This blue fluorescent substance was identified as 2-amino-4-hydroxypteridine-6-aldehyde by its identical chromatographic behaviour in seven different solvent systems with a known specimen of the aldehyde (Table 1). The yield of aldehyde, estimated as before, was about 65% of the theoretical yield from PGA.

DISCUSSION

A reaction scheme for the oxidation of PGA (I) to 2-amino-4-hydroxypteridine-6-carboxylic acid (V) by xanthine oxidase and methylene blue is given on p. 210.

[R represents $HO_2C.[CH_2]_2.CH(CO_2H).NH$–.]

Compounds of the type (I) readily transfer hydrogen to suitable hydrogen acceptors and form anils [corresponding to (II)] which are readily hydrolysed by water to the corresponding amine and aldehyde (Mowat *et al.* 1948). Such dehydrogenation and hydrolysis explains the aerobic alkaline hydrolysis of PGA to 2-amino-4-hydroxypteridine-6-carboxylic acid (Mowat *et al.* 1948) and the sulphurous acid cleavage of PGA to the dihydro-2-amino-4-hydroxypteridine-6-aldehyde (Waller *et al.* 1950). 2-Amino-4-hydroxypteridine-6-aldehyde (IV) is then oxidized to the corresponding carboxylic acid (V) by xanthine oxidase (Lowry *et al.* 1949; Klenow, 1951). This oxidation may also be effected by liver aldehyde oxidase (Kalckar *et al.* 1950).

As the incubation of PGA with xanthine oxidase shows, the enzyme is without effect on PGA. The small amount of carboxylic acid formed in this experiment was derived from the aldehyde present as an impurity in the PGA.

SUMMARY

When pteroyl-L-glutamic acid is incubated with xanthine oxidase and methylene blue it is oxidized to 2-amino-4-hydroxypteridine-6-carboxylic acid. This reaction proceeds by the dehydrogenation and hydrolysis of pteroyl-L-glutamic acid to 2-amino-4-hydroxypteridine-6-aldehyde which is then oxidized by the enzyme to 2-amino-4-hydroxypteridine-6-carboxylic acid.

We wish to thank Professor A. Haddow and Mr R. C. Bray for the gift of xanthine oxidase, and the Chemical Society for a grant.

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Properties of Sickle-Cell Haemoglobin

By A. C. ALLISON
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(*Received* 26 *June* 1956)

Hahn & Gillespie (1927) showed that susceptible erythrocytes are reversibly transformed into sickle cells when deprived of oxygen. Pauling, Itano, Singer & Wells (1949) found that the haemoglobin of patients with sickle-cell anaemia, who are homozygous for the sickle-cell gene, is different from normal adult haemoglobin: in buffers of pH 7·35, I 0·1, the normal molecule has three more negative electronic charges than the abnormal. Sherman (1940) had previously noticed that sickled cells are birefringent, and Pauling and his colleagues inferred that when sickle-cell haemoglobin molecules lose oxygen they combine with one another to form highly anisometric units which become partially aligned within the cell and have enough rigidity to distort it into the sickle shape. In keeping with this interpretation, Harris (1950) reported that stroma-free solutions of sickle-cell haemoglobin of concentration 10 g./100 ml. or higher show a marked increase in viscosity in the absence of oxygen. When such solutions were examined microscopically in the deoxygenated and highly viscous state, they were found to contain birefringent bodies in the shape of double circular cones. These showed all the properties of tactoids or nematic liquid crystals. Harris drew attention to the similarity in shape between these tactoids and sickle cells and concluded that sickle cells are haemoglobin tactoids 'thinly veiled and somewhat distorted by the cell membranes'. On the other hand, Perutz, Liquori & Eirich (1951), having demonstrated that sickle-cell haemogolobin has a much lower solubility than normal haemoglobin and both types of oxyhaemoglobin, suggested that the sickling phenomenon involves crystallization of the haemoglobin in susceptible cells.

The first part of the present investigation has been a more detailed analysis of the physicochemical changes occurring in sickle-cell haemoglobin solutions when they are deoxygenated. It has been possible to show that the conditions required for the formation of a liquid crystalline phase in stroma-free solutions are very similar to those required to induce sickling of susceptible cells.

The increased viscosity and birefringence of concentrated sickle-cell haemoglobin solutions suggests that when they lose oxygen the haemoglobin molecules combine with one another to form highly anisometric polymers. To investigate the part played by hydrogen bonding in this process, the viscosity of sickle-cell haemoglobin has been measured in the presence of urea and of guanidinium ions, agents known to compete for hydrogen bonds. The effect of changes in hydrogen-ion concentration and in the ionic strength of the solvent has also been studied.

Sickling of susceptible erythrocytes is inhibited by Ag^{+}, Hg^{2+} and As^{3+} ions, iodoacetamide, *o*-chloromercuribenzoate and *p*-chloromercuribenzoate, all of which combine with sulphydryl groups (Thomas & Stetson, 1948; Ingbar & Kass, 1951; Allison, unpublished work). These findings suggested to Ingbar & Kass that sulphydryl groups might be specifically concerned in sickling. They then investigated the sulphydryl groups of normal adult and sickle-cell haemoglobin by amperometric titration with silver nitrate, and reported that the normal molecule has two, and that the abnormal molecule has three, available sulphydryl groups. Intermediate values were obtained with specimens from sickle-cell trait carriers, which are mixtures of the two haemoglobin types. Ingram (1955), using a lower ammonia concentration in the silver nitrate titration, concluded that the normal adult haemoglobin molecule has four sulphydryl groups as two pairs of closely neighbouring groups. In the present investigation the effect of various concentrations of agents combining with sulphydryl groups on the viscosity of sickle-cell haemoglobin solutions has been observed, and an attempt has been made to define the way in which such agents inhibit sickling.

Itano & Neel (1950) described another abnormal haemoglobin type, haemoglobin C, which has two negative electronic charges less than sickle-cell haemoglobin in buffers of pH 7·35, I 0·10. The formation of haemoglobin C, normal adult haemoglobin and sickle-cell haemoglobin appears to be controlled by genes at the same locus (Ranney, 1954). Carriers of the sickle-cell trait have sickle-cell and normal adult haemoglobins in their erythrocytes, the former in somewhat greater concentration than the latter (Wells & Itano, 1951). Such cells do not become sickled at physiological oxygen tensions (Allison, 1956). Individuals who inherit both the sickle-cell

and haemoglobin C genes have in their erythrocytes sickle-cell haemoglobin and haemoglobin C in approximately equal proportions: these cells become sickled at relatively high partial pressures of oxygen (Allison, 1956), so that these persons are liable to develop a haemolytic disease known as haemoglobin C–sickle-cell anaemia, the clinical manifestations of which have been described by Smith & Conley (1954) and others. No satisfactory explanation for the much greater susceptibility to sickling of these cells as compared with the cells of sickle-cell trait carriers has been offered. Suggestive observations have, however, been presented by Singer & Singer (1953), who exposed sickle-cell haemolysates to a stream of carbon dioxide gas and recorded the minimum concentration of sickle-cell haemoglobin required for 'gelling'—the development of a solid phase. They reported that the presence of normal adult haemoglobin decreases the minimum concentration of sickle-cell haemoglobin required for gel formation and that haemoglobin C lowers this concentration still further; human foetal haemoglobin had no significant effect. No measurements of viscosity were made, and the pH, ionic strength and temperature of the solutions were not recorded. In attempts to assess the interaction of sickle-cell and other haemoglobin types more precisely, viscosity measurements have been made on a series of mixtures of different haemoglobin types. The results of experiments described below suggest that sickle-cell haemoglobin forms mixed aggregates with normal adult haemoglobin and, more efficiently, with haemoglobin C, and help to explain why subjects with the sickle-cell and haemoglobin C genes are liable to develop haemolysis.

MATERIAL AND METHODS

Blood samples were obtained by venepuncture from four normal adults, four subjects with sickle-cell anaemia (aged 5–18 years), two young adults with haemoglobin C–sickle-cell anaemia, one young adult with haemoglobin C disease (homozygous for the haemoglobin C gene, with all his haemoglobin of type C) and four young adults with the sickle-cell trait. In addition, two samples of umbilical cord blood from infants delivered after pregnancies of 32 and 36 weeks were used.

Preparation of haemoglobin. Concentrated stroma-free solutions of haemoglobin were prepared by washing erythrocytes three times in 0·9% NaCl solution, and lysing the packed cells by repeated freezing and thawing and separation of the stroma by centrifuging at 5000 *g* for 15 min. at 20°. Haemoglobin types were identified by electrophoresis on filter paper in an apparatus similar to that described by Smith & Conley (1954). The proportions of different haemoglobin types in mixed samples were estimated by scanning the paper strips in a densitometer. Replicate experiments gave comparable results and tests with mixtures of known composition were satisfactory, as shown by Larson & Ranney (1953) and Motulsky, Paul & Durrum (1954). Foetal-haemoglobin components were estimated by the denaturation technique of Singer, Chernoff & Singer (1951). These groups of authors present evidence that these two methods of estimating the quantity of various haemoglobin components in mixtures give results which are accurate to $\pm 5\%$ in the range used, which is satisfactory for the present purpose. Foetal haemoglobin represented 84 and 79% of the total in the specimens of umbilical-cord blood and less than 5% in the other specimens. The haemoglobin mixtures used for analysis were natural (in sickle-cell trait carriers and subjects with haemoglobin C–sickle-cell anaemia) and artificially produced by mixing samples of pure haemoglobin types in various proportions. The viscosity measurements obtained with natural and artificial haemoglobin mixtures of the same composition by electrophoretic analysis were identical within the limits of experimental error.

Solutions of haemoglobin of various concentrations were made up in phosphate buffer, ionic strength I 0·01–1·0, pH 6·0–8·0, with K^+ ion as the only cation. The pH was measured with a glass electrode calibrated according to British Standard 1647: 1950. Unless otherwise stated, I was 0·15, pH was 7·35 and experiments were carried out at 37°. The oxyhaemoglobin concentration was measured after dilution in a Beckman spectrophotometer at 541 mμ. before and after each experiment. The oxyhaemoglobin solutions were deoxygenated by evacuation, care being taken to avoid bubbling, and equilibration with $N_2 + CO_2$ (95:5, v/v) for not less than 2 hr. at room temp. (18–19°). Deoxygenation was assumed to be complete when the absorption bands at 541 and 577 mμ. were completely replaced by a band at 555 mμ. Carboxyhaemoglobin was prepared by bubbling CO through haemoglobin solutions, cyanhaemoglobin by adding 6 moles of KCN to each mole of haemoglobin.

Viscosity measurements. These were made in a water bath either by the British Standard Capillary Viscometer (volume 2 ml., flow time 15 sec.) or a falling-ball method. The results with the two techniques were comparable, and the latter was adopted for most of the tests because of its greater convenience. By means of syringes with long needles oxyhaemoglobin specimens were placed in graduated-glass tubes of 10 cm. length and 2·5 mm. bore. Into each tube a steel ball was placed, the diameter of the ball being chosen so that the time of fall through 10 cm. of solvent was approximately 15 sec. at room temp. The tube was sealed with a silicone-treated rubber stopper, and the time taken for the ball to fall through 10 cm. of the solution was recorded at various temperatures. The specimens were then deoxygenated and cooled to 4°, at which temperature they were all fluid and could be placed in the viscometer tubes. The experiments were then repeated with the solvent only in the tubes.

Specific viscosity ($\eta_{sp.}$) was then calculated from the equation:

$$\eta_{sp.} = \frac{\eta - \eta_0}{\eta_0},$$

where η is the viscosity of the solution and η_0 is the viscosity of the solvent alone.

EXPERIMENTAL

The viscosity of normal adult and sickle-cell oxyhaemoglobin, haemoglobin, carboxyhaemoglobin and cyanhaemoglobin solutions varying in concentration from 0·05 to 25 g./100 ml. was measured over the temperature range 0–40°. Concentrated solutions of sickle-cell haemoglobin

were examined under the polarizing microscope in preparations made by sealing the coverslip to the microscope slide with paraffin wax.

The viscosity of sickle-cell haemoglobin solutions was then measured in the presence of urea and guanidinium chloride. The solutions of sickle-cell haemoglobin contained 14·6 and 16·4 g. of haemoglobin/100 ml. and the concentrations of urea and guanidinium chloride were 0·01–1 M and 0·005–0·5 M respectively. The viscosity of concentrated sickle-cell haemoglobin solutions made up in a series of nine phosphate buffers (pH 6·0, 7·35 and 8·0, I 0·01, 0·1 and 1·0) was then measured at 37°. Known molar concentrations of $AgNO_3$, $HgCl_2$ and sodium p-chloromercuribenzoate were added to sickle-cell oxyhaemoglobin solutions, the concentrations of which were 14·6 and 16·4 g./100 ml. The haemoglobin specimens had been previously dialysed against a large volume of buffer for 24 hr. at 4° to eliminate glutathione. After 30 min. the viscosity of the oxyhaemoglobin solutions was measured; then the solutions were deoxygenated and the viscosity was measured again.

Finally, the viscosity of various oxyhaemoglobin and haemoglobin mixtures, both natural (from sickle-cell trait carriers and subjects with haemoglobin C–sickle-cell anaemia) and prepared artificially by mixing pure haemoglobin types in various proportions, was measured at 37°. The solutions were made up in phosphate buffer, I 0·15, pH 7·35. Two points were recorded for each mixture: the minimum concentration of sickle-cell haemoglobin required for the development of a uniformly liquid crystalline phase, and that required to produce an increment in viscosity on deoxygenation (i.e. the minimum concentration of sickle-cell haemoglobin at which aggregation of the haemoglobin molecules may be assumed to take place). The recorded results refer to mixtures of sickle-cell (S) haemoglobin with foetal (F) and normal adult (A) haemoglobins and haemoglobin C. Mixtures without sickle-cell haemoglobin show no departure from the normal viscosity curve, either oxygenated or deoxygenated, so that the results have not been presented in detail.

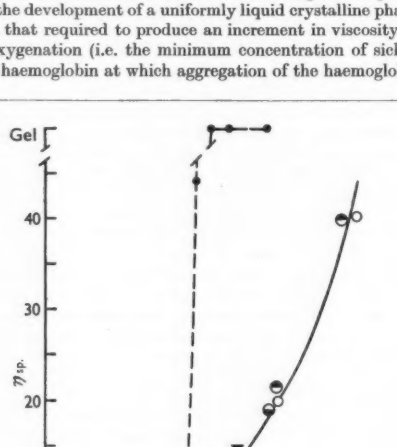

Fig. 1. Specific viscosity of haemoglobin solutions as a function of concentration. ○, Normal adult haemoglobin; ◓, normal adult oxyhaemoglobin; ●, sickle-cell haemoglobin; ◒, sickle-cell oxyhaemoglobin; ▲, sickle-cell cyanhaemoglobin; ▼, sickle-cell carboxyhaemoglobin.

RESULTS

Effect of concentration and temperature on the viscosity of normal adult and sickle-cell haemoglobin and oxyhaemoglobin solutions. The results of viscosity measurements of various haemoglobin solutions are shown graphically in Figs. 1 and 2. The specific

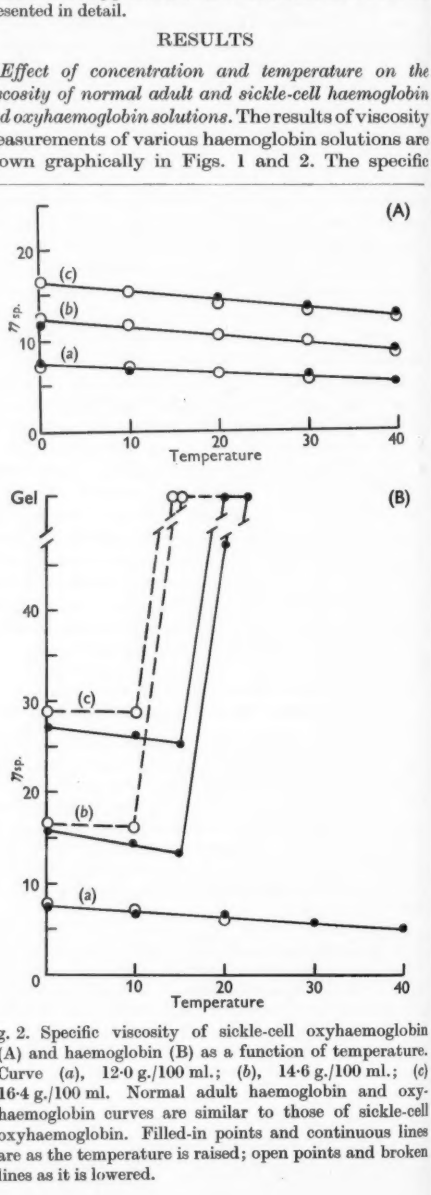

Fig. 2. Specific viscosity of sickle-cell oxyhaemoglobin (A) and haemoglobin (B) as a function of temperature. Curve (*a*), 12·0 g./100 ml.; (*b*), 14·6 g./100 ml.; (*c*) 16·4 g./100 ml. Normal adult haemoglobin and oxyhaemoglobin curves are similar to those of sickle-cell oxyhaemoglobin. Filled-in points and continuous lines are as the temperature is raised; open points and broken lines as it is lowered.

viscosity of normal adult haemoglobin solutions increases as the concentration rises; at any one concentration the viscosity falls as the temperature is raised from 0° to 40°. Normal adult oxyhaemoglobin and sickle-cell oxyhaemoglobin solutions give identical results, in agreement with the observations of Nakamura (1955) on dilute oxyhaemoglobin solutions. Sickle-cell haemoglobin solutions in concentrations up to 12 g./100 ml. have also the same viscosity as normal adult haemoglobin. Sickle-cell haemoglobin solutions of higher concentration, however, show a marked increase in viscosity. At 0° the specific viscosity of such solutions is significantly greater than that of corresponding normal adult haemoglobin solutions. The same order of difference is maintained up to 15°; above this temperature the specific viscosity of the concentrated sickle-cell haemoglobin solutions show a further sharp increase until at 20° transformation into a solid, gel-like mass takes place. When the solutions are cooled the sequence is reversed, the system showing marked hysteresis. The observation can be repeated several times on the same solution.

The viscosity of sickle-cell carboxyhaemoglobin and cyanhaemoglobin is indistinguishable from that of oxyhaemoglobin; and, as is well known, addition of carbon monoxide or cyanide to susceptible cells prevents sickling when they are exposed to atmospheres of nitrogen.

Formation of tactoids in sickle-cell haemoglobin solutions. The solid material obtained by deoxygenating concentrated sickle-cell haemoglobin solutions was found to be birefringent. With high concentrations of sickle-cell haemoglobin the birefringence was uniform, but with lower concentrations of pigment (12·5–16 g./100 ml.) or in partially oxygenated solutions, birefringent tactoids separated by isotropic material were observed. The tactoids had the form of spindles or double circular cones, 1–5 μ. long and 0·2–1 μ. wide, and were very similar to those described and figured by Harris (1950). When solutions containing tactoids were centrifuged at 4000 *g* for 30 min. at 20° two phases were separated. The concentration of haemoglobin in the upper, liquid phase was 12·6 g./100 ml., and that in the lower, solid phase was 16·5 g./100 ml.

Preliminary investigations showed that the phase rule is, in general, obeyed: raising the haemoglobin concentration between 12·6 and 16·5 g./100 ml. increases the size and number of tactoids visible in microscopical preparations and increases the volume of the solid phase at the expense of the liquid phase without significantly affecting the composition of either. However, this result is obtained only when oxyhaemoglobin solutions of various concentrations are deoxygenated at a constant temperature. A higher proportion of the solid phase is obtained by diluting concentrated haemoglobin solutions to the same final concentration, presumably because of hysteresis. The system is profoundly affected by changes in temperature and by the presence of small amounts of oxygen.

Effect of urea and guanidinium chloride on the viscosity of sickle-cell haemoglobin solutions. From the results shown in Fig. 3 it seems that in the presence of relatively low concentrations of urea and guanidinium chloride (0·01 and 0·005 M respectively) the increment in viscosity which takes place when sickle-cell haemoglobin solutions are deoxygenated is not affected. However, in the presence of higher concentrations of urea and guanidinium chloride (M and 0·5 M respectively) no viscosity increment at all was observed when the solutions were deoxygenated, and with intermediate concentrations (0·1 M urea and 0·05 M guanidinium chloride) the viscosity increment was present but reduced in magnitude.

Effect of H^+-ion concentration and ionic strength on viscosity of sickle-cell haemoglobin solutions. The viscosity increment on deoxygenation was of the same order in all the buffers used, from which it appears that changes in H^+-ion concentration and

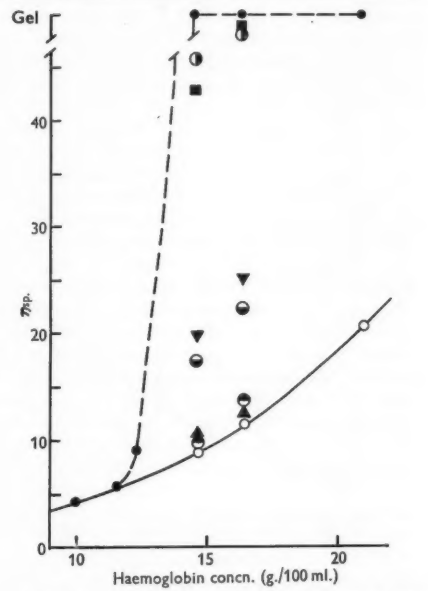

Fig. 3. Specific viscosities. ●, Sickle-cell haemoglobin; ○, sickle-cell oxyhaemoglobin; ◑, sickle-cell haemoglobin (S) in the presence of 0·01 M urea; ◒, S in 0·1 M urea; ◓, S in M urea; ■, S in 0·005 M guanidinium chloride; ▼, S in 0·05 M guanidinium chloride; ▲, S in 0·5 M guanidinium chloride.

ionic strength in the range tested do not significantly affect aggregation of the sickle-cell haemoglobin molecules. Field & O'Brien (1955) report that the sedimentation and diffusion of human carboxyhaemoglobin in 0·1 M buffers are the same between pH 6 and 11 and with added sodium chloride concentrations of up to M.

Effect of agents combining with sulphydryl groups on the viscosity of sickle-cell haemoglobin. From the results of the experiments shown in Fig. 4 it seems that the viscosity of the sickle-cell oxyhaemoglobin solutions is not affected by the presence of Ag^+, Hg^{2+} and *p*-chloromercuribenzoate ions; when the concentration of these ions is high enough, however, no increment in viscosity takes place on deoxygenation. The critical concentration of Ag^+ ions (four per molecule of haemoglobin) is greater than that of Hg^{2+} or *p*-chloromercuribenzoate ions (two per molecule).

Effect of other haemoglobin types on the viscosity of sickle-cell haemoglobin solutions. The results of this series of experiments are summarized in Fig. 5. It is clear that the presence of foetal haemoglobin has no significant effect on the concentration of sickle-cell haemoglobin required for the development of a solid phase or for a viscosity increment to occur on deoxygenation; the small effect observed could be due to the presence of minor components of normal adult haemoglobin in the foetal haemoglobin specimens used. In the presence of normal adult haemoglobin or haemoglobin C, however, the concentration of sickle-cell haemoglobin required for both these changes is diminished. Normal adult haemoglobin is nearly as effective as haemoglobin C in mixtures where the proportion of sickle-cell haemoglobin is about 75% or higher; when the proportion of normal adult haemogolobin or haemoglobin C is raised to above 25%, haemoglobin C is much more effective in producing viscosity increments than normal adult haemoglobin. Indeed, mixtures of equal quantities of sickle-cell haemoglobin and haemoglobin C show viscosity changes on deoxygenation nearly as great as those shown by pure sickle-cell haemoglobin solutions of the same total haemoglobin concentration. Raising the proportion of haemoglobin C above 50% seems to have very little, if any, additional effect.

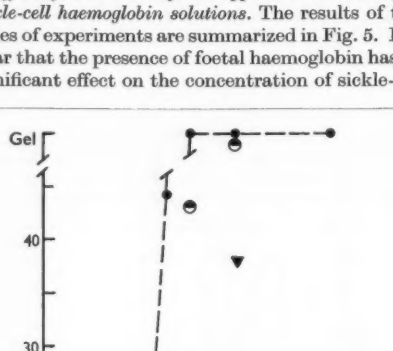

Fig. 4. Specific viscosities. ●, Sickle-cell haemoglobin; ○, sickle-cell oxyhaemoglobin; ◓, sickle-cell haemoglobin (S) in the presence of 1 Ag^+ ion/mole; ◑, S plus 2 Ag^+ ions/mole; ◒, S plus 4 Ag^+ ions/mole; ▼, S plus 1 Hg^{2+} ion/mole; ▲, S plus 2 Hg^{2+} ions/mole; ◆, S plus *p*-chloromercuribenzoate (1 mole/mole); ■, S plus 2 moles of *p*-chloromercuribenzoate/mole.

DISCUSSION

There has been much speculation about the way in which deoxygenation of sickle-cell haemoglobin brings about the characteristic change in shape of

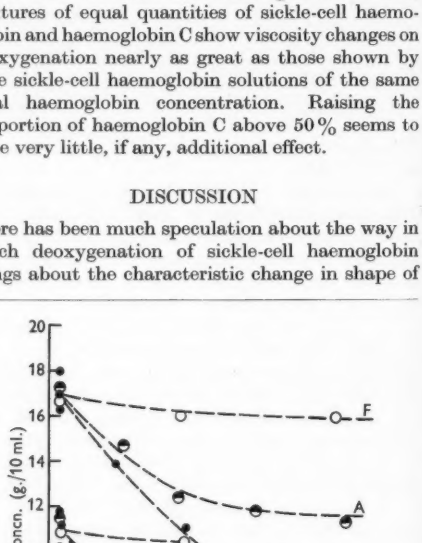

Fig. 5. Concentration of sickle-cell haemoglobin (S) in mixtures with human foetal (F) and normal adult (A) haemoglobin and haemoglobin C required for the formation of a liquid crystalline phase (upper set of curves) and for a significant viscosity increment on deoxygenation (lower set of curves).

susceptible erythrocytes. In the paper announcing the discovery of sickle-cell haemoglobin, Pauling *et al.* (1949) proposed that there is a surface region on the globin of the sickle-cell haemoglobin molecule which is absent in the normal molecule and which has a configuration complementary to a different region of the surface of the haemoglobin molecule. The fact that sickling occurs only when the partial pressures of oxygen are low suggested to Pauling that the combining site is near the iron atom of one or more of the haems, and that when the iron atom is combined with either oxygen or carbon monoxide the complementary nature of the two structures is considerably diminished. When they lose oxygen, the sickle-cell haemoglobin molecules might be capable of interacting with one another at these sites sufficiently to cause at least a partial alignment of the molecules within the cell, so that the cell membrane becomes distorted to accommodate the now relatively rigid structure within its confines.

The development of complementary configuration in deoxygenated sickle-cell haemoglobin molecules might be due to the removal of sterically interfering oxygen or carbon monoxide molecules, as suggested by Pauling and his colleagues; or it might be due to the changes in molecular configuration which are known to accompany deoxygenation. These changes are reflected in the marked differences between the crystal forms of human adult haemoglobin and oxyhaemoglobin (Perutz & Weisz, 1947), and in the altered magnetic susceptibility of haemoglobin on deoxygenation (Pauling & Coryell, 1936), which suggests that the bonds between the haem prosthetic group and globin have changed from the essentially covalent to the essentially ionic type. This must, in turn, involve a secondary change in the electronic configuration of the globin molecule in the vicinity of the point of attachment of the haem groups, and the existence of such a change is made probable by the double-peaked fluctuation in the dielectric increment of haemoglobin in the course of oxygenation (Takashima, 1956).

Removal of oxygen from normal adult haemoglobin or haemoglobin C molecules, or sickle-cell haemoglobin molecules in dilute solutions, has no significant effect on their specific viscosity, which suggests that deoxygenation produces no large alteration in the hydration, partial specific volume or axial ratio of the molecules. The development of birefringence and increase in viscosity in concentrated solutions of sickle-cell haemoglobin when they are deoxygenated is probably due to aggregation of the nearly isometric molecules into highly anisometric polymers. Since this process of aggregation occurs only in concentrated solutions, is reversibly dependent on temperature and is easily reversed by addition of oxygen or carbon monoxide, it can be concluded that the intermolecular attractive forces are relatively weak. This interpretation is confirmed by the ease with which aggregation of the sickle-cell haemoglobin molecules can be prevented by the presence of relatively low concentrations of urea and guanidinium ions. This is indirect evidence that hydrogen bonding is involved in the process, and is probably reinforced by other low-energy bonds of the type existing between molecules of antigens and antibodies (Pauling, 1946). The aggregation of sickle-cell haemoglobin molecules is unaffected by changes in ionic strength and H^+-ion concentration over a relatively wide range, from which it seems unlikely that salt linkages play an important part in the reaction.

In view of the dependence of the reaction on temperature, and the influence on it of urea and guanidinium ions, it is also very improbable that disulphide bonds are formed between the molecules. The prevention of sickling of susceptible erythrocytes, or aggregation of sickle-cell haemoglobin molecules, by agents combining with sulphydryl groups is probably not a specific effect, as was concluded by Ingbar & Kass (1951). However, attachment by covalent bonds of molecules such as *p*-chloromercuribenzoate to sickle-cell haemoglobin molecules might well interfere sterically with the close apposition of the latter required for stable low-energy combination. This suggests that the potential combining sites on the surface of sickle-cell haemoglobin molecules are in the vicinity of the available sulphydryl groups. Relevant in this connexion is the recent report of Riggs & Wolbach (1956) that the addition of two molecules of mersalyl, mercuric chloride and *p*-chloromercuribenzoate to each mole of horse haemoglobin greatly reduces the haem–haem interaction in oxygenation. From their results Riggs & Wolbach conclude that the two haems in each half-molecule of horse haemoglobin are situated on either side of a cluster of SH groups. The observations given in the present paper, showing that aggregation of sickle-cell haemoglobin molecules can be prevented by two moles of mercuric chloride or *p*-chloromercuribenzoate per mole of haemoglobin, as well as by attachment of oxygen, carbon monoxide or cyanide to haem groups, suggest that in human haemoglobin, also, the available sulphydryl groups may be in the vicinity of the haem groups.

It has been noted that deoxygenation of concentrated sickle-cell haemoglobin solutions at low temperatures (0–15°) produces small, but consistent, increases in viscosity, whereas deoxygenation at higher temperatures produces large increases in viscosity. One interpretation of this finding is the formation of finite polymers at low temperatures and of high polymers when the temperature is increased above 15°. Another remarkable finding has been the effect of other haemoglobin types on the

aggregation of sickle-cell haemoglobin molecules. Foetal haemoglobin has no detectable effect on the process, from which it seems that mixed aggregates are not formed. This is to be expected, since normal human adult and foetal haemoglobins appear to have completely different configurations, whereas the close similarity in configuration of normal adult and sickle-cell oxyhaemoglobin molecules is emphasized by the identity of the X-ray diffraction patterns of most of their crystal forms (Perutz *et al.* 1951).

In the absence of sickle-cell haemoglobin no aggregation occurs at all. It is therefore inferred that the sickle-cell haemoglobin molecule is the only one that possesses the combining site when deoxygenated. This site is, however, complementary to the corresponding regions of normal adult haemoglobin and haemoglobin C, as well as other sickle-cell haemoglobin, molecules. In polymers, then, at least every second molecule must be of the sickle-cell type. The fact that haemoglobin C is able to replace sickle-cell haemoglobin in mixtures where the proportion of haemoglobin C is as high as, but not higher than, 50 % suggests that mixed polymers may be of the type SCSCSC.... Normal adult haemoglobin, however, is capable of replacing sickle-cell haemoglobin in mixtures where the proportion of the former is not greater than about 25 %, from which it seems that the mixed polymers may be of the type SSSASSSA.... A possible explanation for this type of aggregation is offered below. The greater efficiency of formation of mixed aggregates with haemoglobin C than with normal adult haemoglobin explains the finding that erythrocytes from subjects with haemoglobin C–sickle-cell anaemia become sickled at higher partial pressures of oxygen than cells from carriers of the sickle-cell trait (Allison, 1956). This, in turn, explains the liability of the former but not the latter to develop haemolytic disease.

These two properties of sickle-cell haemoglobin—the formation of finite polymers at low temperatures and the greater efficiency of formation of mixed aggregates with haemoglobin C than with normal adult haemoglobin—can be explained by assuming that the asymmetric aggregates are helical and not linear. This type of aggregation of proteins has been discussed by Pauling (1953), who points out that if slightly asymmetric globular molecules combine with one another the resulting aggregates may be helical, and that these might easily form liquid crystals of the nematic type. He also notes that if the pitch of the helix is less than the width of the molecule in the direction of the axis of the helix, steric hindrance will prevent the formation of a fibril; the process of aggregation resulting from the combination of complementary surfaces would then result in the formation of finite polymers. This process might well occur in sickle-cell haemoglobin solutions at low temperatures, whereas complete fibrils are formed at higher temperatures. In the course of formation of mixed aggregates of sickle-cell and normal adult haemoglobin, steric hindrance might prevent the attachment of a molecule of the latter to every sickle-cell haemoglobin molecule in the helix, so that an SSSASSSA... pattern of combination, and not an SASASA... pattern, is produced. Further observations on the lateral dimensions of the aggregates should establish whether they are, in fact, helices.

However the anisometric aggregates are formed, it seems clear that they attract one another into a parallel orientation to produce a liquid crystalline phase. When the sickle-cell haemoglobin concentration is between 12·6 and 16·5 g./100 ml., spindle-shaped, birefringent, liquid crystalline masses (tactoids) are separated by haemoglobin molecules in a state of low order. These are very similar to tactoids seen in other systems, e.g. the tobacco-mosaic virus (Bernal & Fankuchen, 1941). When the sickle-cell haemoglobin concentration is above 16·5 g./100 ml. the entire haemoglobin mass is in the liquid crystalline phase. It may be noted that sickled cells are completely liquid crystalline masses, separated by cell membranes, not tactoids separated by haemoglobin in a state of low order, as supposed by Harris (1950) and others. It has been shown by Allison (1956) that the conditions required for sickling of susceptible erythrocytes are very similar to those required for the formation of a liquid crystalline phase in sickle-cell haemoglobin solutions *in vitro*. Hence there is little doubt that the two processes are the same, and that the erythrocyte membrane and stroma play little direct part in the sickling phenomenon.

SUMMARY

1. The specific viscosity of sickle-cell haemoglobin has been measured under a variety of conditions. Sickle-cell oxyhaemoglobin shows the same type of viscosity behaviour as normal adult oxyhaemoglobin and haemoglobin. The viscosity rises steadily as the concentration is increased from 0·5 to 25 g./100 ml. and falls at any one concentration as the temperature is increased from 0° to 40°. When the concentration of sickle-cell haemoglobin is raised above 12 g./100 ml. the viscosity increases very much more rapidly than that of normal adult haemoblogin, until at 16 g./100 ml. a completely rigid, birefringent, liquid crystalline phase is formed. When the concentration of the solution is between these two critical points, spindle-shaped liquid crystalline masses or tactoids, separated by haemoglobin in a state of low order, are observed.

2. When concentrated sickle-cell oxyhaemoglobin solutions are deoxygenated at low temper-

atures (0–15°) the viscosity increment is small, from which it is concluded that finite polymers are formed. When the temperature is raised the usual liquid crystalline phase develops, and this disappears when the solution is cooled.

3. The increment in viscosity usually observed when concentrated sickle-cell oxyhaemoglobin solutions are deoxygenated does not occur in the presence of M urea or 0·5M guanidinium chloride, but is unaffected by changes in pH from 6·0 to 8·0 or changes in the ionic strength of the solvent from 0·01 to 1·0. It is inferred that hydrogen bonding plays an important part in aggregation of sickle-cell haemoglobin molecules, and formation of salt linkages only a minor part.

4. Sickling of susceptible cells, and aggregation of sickle-cell haemoglobin molecules, do not take place in the presence of Ag^{+}, Hg^{2+} and *p*-chloromercuribenzoate ions in concentrations of four, two and two respectively per molecule of haemoglobin. It is concluded that these compounds form covalent bonds with exposed sulphydryl groups in the vicinity of the complementary sites where sickle-cell haemoglobin molecules normally combine with one another (probably close to the haem groups), so that by steric hindrance the combination is prevented.

5. Measurements of the viscosity of haemoglobin mixtures suggest that sickle-cell haemoglobin forms mixed aggregates with normal adult haemoglobin and haemoglobin C, but not with human foetal haemoglobin. It seems that haemoglobin C can replace more molecules of sickle-cell haemoglobin in the mixed aggregates than can normal adult haemoglobin. This finding, and the formation of finite sickle-cell haemoglobin polymers at low temperatures, can be explained by postulating a helical rather than a linear process of aggregation of sickle-cell haemoglobin molecules when they are deoxygenated.

I am indebted to many colleagues, in particular Dr G. Carpenter, Los Angeles, Dr H. M. Ranney, New York, and Dr G. M. Edington, Accra, for supplying blood specimens from patients with sickle-cell anaemia and its genetic variants. The *p*-chloromercuribenzoic acid used was kindly given by Dr L. A. Stocken, Oxford. The investigation was begun in the Gates and Crellin Laboratory, California Institute of Technology, during the tenure of an Eli Lilly Travelling Fellowship; and I wish to thank Professor L. Pauling and Drs J. Vinograd and M. Murayama for their interest and help in this stage of the work. I am grateful also to Professor H. A. Krebs and Drs A. G. Ogston and R. Cecil, Oxford, for their advice in the preparation of the manuscript for publication.

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Biosynthesis of Ergothioneine by *Claviceps purpurea*

2. INCORPORATION OF [^{35}S]METHIONINE AND THE NON-UTILIZATION OF [2(RING)-^{14}C]HISTAMINE*

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The incorporation of [2-^{14}C]acetate into ergothioneine by *Claviceps purpurea* has been previously reported by Heath & Wildy (1956*a*, *b*). This work showed that there was a close relationship between histidine and ergothioneine biosynthesis. Histidine might be converted directly into ergothioneine in the fungus or alternatively ergothioneine might be derived from a compound closely related structurally to histidine.

Since histamine occurs in ergot (Barger & Dale, 1910) and its conversion into ergothioneine could be envisaged, it was decided to test this substance as an ergothioneine precursor in our system, as [2(ring)-^{14}C]histamine was immediately available and [2(ring)-^{14}C]histidine was not available to us at the time. Studies with [2-^{14}C]histidine will be reported later. If ergothioneine is formed directly from histidine, then in order that the [2-^{14}C]histamine should be incorporated into the ergothioneine, it would be necessary for it to be first carboxylated and this would result in the general pool of histidine in the fungus being labelled. If, on the other hand, ergothioneine is formed from histidine by the partial degradation of the side chain, such as to imidazole-ethanol, which could also be formed by deamination of histamine, followed by a condensation to yield the betaine of ergothioneine, then labelled ergothioneine could be derived from [2-^{14}C]histamine without the formation of labelled histidine. The same result would be obtained if histamine derived by decarboxylation of histidine were methylated and subsequently recarboxylated.

Sulphate can provide the sulphur for ergothioneine in this system (Heath & Wildy, 1956*b*) but nothing is known of the immediate sulphur precursors. In order to elucidate further this aspect of the problem, the incorporation of [^{35}S]methionine was studied.

Some of these results have been presented in a preliminary form (Wildy & Heath, 1956).

EXPERIMENTAL

Organism. The culture of *Claviceps purpurea* no. 44613 was originally obtained from the Commonwealth Mycological Institute, Kew, London, and was maintained as described previously (Heath & Wildy, 1956*b*).

[2(ring)-^{14}C]Histamine and DL-*[^{35}S]methionine.* These were obtained from the Radiochemical Centre, Amersham. The activity of the histamine dihydrochloride was 4·7 mc/m-mole. At the commencement of the methionine experiment 5 mg. of DL-methionine gave $1{\cdot}24 \times 10^7$ counts/min. counted at infinite thinness with a thin mica end-window Geiger tube.

The following materials and methods used have been previously described (Heath & Wildy, 1956*b*): activated alumina; Zeo-Karb 225; Oxoid Membrane Filters; sterilization; culture media; preparation of inoculum; detection and estimation of ergothioneine.

Growth of Claviceps purpurea *on a medium containing* [2-^{14}C]*histamine.* Five 120 ml. shake cultures were grown in 350 ml. Erlenmeyer flasks. Two of these were controls containing only the standard mannitol–ammonium succinate medium; one flask was supplemented with 0·1 mc of [2-^{14}C]histamine dihydrochloride (3·9 mg.); 0·1 ml. of this medium was removed before sterilization in order to determine the total activity present, as measured by our apparatus; the remaining two flasks were each supplemented with 3·9 mg. of unlabelled histamine dihydrochloride. All were inoculated with 1 ml. of conidial suspension and the cultures were grown simultaneously at room temperature for 10 days.

The total growth (mycelium and conidia) was harvested separately from each flask by filtration on Oxoid membranes and washing with water. The filtrate and washings from the [2-^{14}C]histamine flask were made up to 250 ml. and a portion was used to determine the activity remaining in the culture broth.

Ergothioneine was estimated in the control growth to ensure that the fungus was still producing ergothioneine (at one stage in this work the fungus failed to produce ergothioneine and it was always necessary to check with control growth). In one histamine control, estimation of ergothioneine was found to be impracticable owing to interference with the Hunter-colour reaction by other diazo-reacting compounds, which were not separated by the alumina chromatography. The presence of ergothioneine was definitely established by paper chromatography of the eluate from the alumina column. From the appearance of the chromatogram it was apparent that the usual amount of ergothioneine was present.

Isolation of ergothioneine from the [2-^{14}C]*histamine culture.* L-Ergothioneine hydrochloride (90 mg.) was added to the total growth obtained from the [2-^{14}C]histamine experiment, which was then extracted three times with water (total vol. 40 ml.) by immersion in boiling water. The residue was then extracted twice by boiling with water (total vol. 40 ml.). The

* Part 1: Heath & Wildy (1956*b*).

combined extracts were deproteinized and chromatographed on Zeo-Karb 225 (4½% cross-linked) and alumina as described (Heath & Wildy, 1956*b*) for the isolation of [^{14}C]ergothioneine from the growth of *C. purpurea* on [2-^{14}C]acetate. In the present case, however, the ergothioneine-containing fractions from the alumina column were taken to dryness and rechromatographed on a second alumina column and finally on a Zeo-Karb 225 column. The resulting ergothioneine, after extraction with ethanol, weighed 63·5 mg. It was recrystallized seven times and its radioactivity measured after each recrystallization.

Isolation of histidine from the [2-^{14}C]*histamine culture.* The residue of the fungus after aqueous extraction was hydrolysed by refluxing in 6N-HCl for 20 hr., evaporated to dryness and finally dried in a vacuum desiccator over NaOH. The residue was dissolved in water and filtered. One-dimensional chromatograms, run in butanol–acetic acid, showed that a general distribution of amino acids, including histidine, was present. Also, a radioactive spot with R_F slightly greater than histidine was detected. The main bulk of the filtrate was brought to pH 4 with 2N-NaOH, and L-histidine hydrochloride monohydrate (250 mg.) was added, followed by saturated ethanolic $HgCl_2$ (10 ml.); the pH was adjusted to 7·2 with saturated $NaHCO_3$ to precipitate the mercury complex, and the mixture was allowed to stand at 4° for 1 hr. The precipitate was filtered off, washed with water and decomposed with H_2S. The HgS was removed by filtration, the filtrate was concentrated to 25 ml. and 3:4-dichlorobenzenesulphonic acid (1 g.) was added to the hot solution. After standing overnight at 4° the crystals were filtered off and washed with ice-cold water; wt. 450 mg. It was recrystallized five times and the radioactivity of the crystals was measured each time.

Labelled compounds remaining in culture broth after growth of Claviceps purpurea *on* [2-^{14}C]*histamine.* The filtrate and washings obtained during the harvesting of the fungus grown on [2-^{14}C]histamine were passed through a 15 cm. × 0·9 cm. column of the H^+ form of Zeo-Karb 225, 4½% cross-linked, 100–150 mesh. All the radioactivity was retained by the column which, after washing with water, was eluted with 0·1N-NH_3 soln. and the radioactive fractions were combined and evaporated to dryness. The residue was dissolved in 0·4 ml. of water and 10 μl. portions were subjected to ascending chromatography in *n*-butanol saturated with 6N-NH_3 soln. (a solvent which effectively separates histamine from histidine; Urbach, 1949). The chromatograms were developed with ninhydrin, Pauly diazo reagent and dichloroquinonechloroimide, and scanned with an end-window Geiger counter.

A volume (50 μl.) of the solution was mixed with 50 μl. of 12N-HCl and hydrolysed by heating at 100° for 24 hr. in a sealed capillary. The dried residue was dissolved in water and chromatographed in ascending butanol–NH_3 soln. and descending butanol–acetic acid.

Growth of Claviceps purpurea *on a medium containing* [^{35}S]*methionine.* DL[^{35}S]Methionine (5 mg.) was added to 120 ml. of the standard mannitol–ammonium succinate medium in a 350 ml. Erlenmeyer flask, inoculated with 1 ml. of conidial suspension and grown for 10 days. Ergothioneine was isolated from the total growth by extraction with water, ethanol deproteinization and alumina chromatography. The [^{35}S]ergothioneine thus obtained was crystallized to constant activity after conversion into ergothioneine tartrate.

RESULTS

The activity at infinite thinness of the [2-^{14}C]-histamine medium before growth was $1{\cdot}58 \times 10^7$ counts/min. and after growth the culture broth gave $1{\cdot}04 \times 10^7$ counts/min.; hence 66% of the isotope was not incorporated into the total growth.

There was no incorporation of isotope into either the isolated histidine or ergothioneine, but the metabolites of histamine were not easily separated from either histidine or ergothionine either on ion-exchange or alumina columns. This separation was achieved only by repeated crystallizations, as shown in Table 1.

The results of scanning the paper chromatograms showed that activity was associated only with diazo-reacting compounds which were still highly labelled, giving counts on the chromatogram of the order of 3000–6000 counts/min. Free histamine was no longer present in the culture broth; after hydrolysis, however, radioactive histamine was detected, probably derived from an acylated histamine. The histidine spot was not radioactive, showing that carboxylation of histamine to histidine had not occurred. One very radioactive spot was identical with imidazoleacetic acid, both in butanol–ammonia and butanol–acetic acid. From radioautographs of the chromatograms three other diazo-reacting metabolites of histamine were detected. No radioactivity was associated with ninhydrin-reacting substances. It appears, therefore, that the metabolism of histamine by this fungus brings about the degradation of the side chain but does not break down the imidazole ring.

The ergothioneine isolated from the culture of *C. purpurea* grown on [^{35}S]methionine had an activity of 3542 counts/min. for 1 cm.^{2} at infinite

Table 1. *Decrease in radioactivity of ergothioneine and histidine bis-3:4-dichlorobenzenesulphonate isolated from a culture of* Claviceps purpurea *grown on a medium containing* [2-^{14}C]*histamine, on crystallization from aqueous ethanol and water respectively*

Activities are given as counts/min., at infinite thickness of 1 cm.^{2} areas.

	Activities	
Crystallization	Histidine bis-3:4-dichloro-benzenesulphonate	Ergothioneine
1st	3315	1274
2nd	1264	610
3rd	400	205
4th	147	140
5th	50	63
6th	8	39
7th	—	32
8th	—	20

thickness after crystallization, to constant radioactivity, from water as the tartrate. Scanning of paper chromatograms revealed only one radioactive spot, which coincided with ergothioneine.

DISCUSSION

As can be seen from the results, neither the ergothioneine nor the histidine isolated from a culture of *Claviceps purpurea* grown on a medium containing [2-^{14}C]histamine was labelled. In previous communications (Heath & Wildy, 1956*a*, *b*) it has been shown by the incorporation of [2-^{14}C]acetate that there is a direct relationship between the biosynthesis of ergothioneine and histidine in this system. It is obvious from the present results that the carboxylation of histamine does not take place. This is not unexpected since the carboxylation of amines to yield amino acids is not a normal biosynthetic pathway, although certain examples of the reversibility of amino acid decarboxylases have been reported (Hanke & Siddiqi, 1950). Although primary amines such as aminoethanol (Horowitz, 1946), noradrenaline (Bülbring, 1949) and tyramine (Leete, Kirkwood & Marion, 1952) have been shown to be methylated in biochemical processes, the possibility that the betaine of ergothioneine is formed by the methylation of histamine followed by carboxylation is ruled out by the above results. The methylation of amino acids, on the other hand, is not a normal biosynthetic process, but some method must exist for the methylation by which the betaine structure of ergothioneine is built up. By analogy with the biosynthesis of betaine, which is formed by the methylation of aminoethanol to give choline with subsequent oxidation to yield betaine and not by the direct methylation of glycine, it might be expected that ergothioneine would be formed by the introduction of methyl groups at the histidinol stage with subsequent oxidation to the betaine. Such a pathway, moreover, would be consistent with our earlier results which show an identical pattern of labelling in both histidine and ergothioneine derived from [2-^{14}C]acetate.

The present work has also thrown light on the metabolism of the imidazole-ring system by this fungus, because the activity remaining in the culture medium was associated with imidazole compounds and there appeared to be no degradation of the imidazole ring to yield labelled fragments. There was no detectable radioactivity in any of the amino acids, and the fraction of the culture broth not absorbed on the Zeo-Karb 225 column, which would contain the carboxylic acids (including formate), was completely inactive. Free histamine no longer occurred, although some could be liberated on acid hydrolysis which was probably derived from an acylated histamine such as acetylhistamine. Oxidation of the side chain had occurred, as shown by the presence of imidazoleacetic acid and the other radioactive diazo-reacting substances. Recently Karjala, Turnquest & Schayer (1956) have shown that the metabolites of histamine in mouse urine consist of 1-methylimidazole-4-acetic acid, 1-methylimidazole-5-acetic acid and 1-ribosylimidazole-4(5)-acetic acid, as well as imidazoleacetic acid. The fission of the imidazole ring of imidazoleacetic acid to yield formylaspartic acid, as shown to occur in *Pseudomonas* by Hayaishi (1955), did not appear to take place in this fungus.

Claviceps purpurea has been shown to be capable of utilizing the sulphur of methionine for the synthesis of ergothioneine. The actual pathway of incorporation is being further studied to determine the direct sulphur precursor, as these results do not necessarily show that methionine itself is a direct sulphur donor.

SUMMARY

1. [2-^{14}C]Histamine was not incorporated into either ergothioneine or histidine by *Claviceps purpurea*.

2. The imidazole ring of histamine was not degraded by this fungus and the residual activity detected was associated only with imidazole compounds. No activity was present in either amino or carboxylic acids.

3. [^{35}S]Ergothioneine was isolated from a culture of *Claviceps purpurea* grown on a medium containing [^{35}S]methionine.

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Studies on Ali-esterases

5. SUBSTRATE SPECIFICITY OF THE ESTERASES OF SOME SAPROPHYTIC MYCOBACTERIA*

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Recent investigations by Mendel, Myers, Uyldert, Ruys & de Bruyn (1953) and by Ruys (1954) have shown that the growth of *Mycobacterium tuberculosis* can be inhibited by relatively small amounts of various organophosphorus compounds under specified conditions. A similar finding had been briefly reported for *Lactobacillus arabinosus* (C. Haber, quoted by Brooks, Ransmeier & Gerard, 1949). However, Mendel *et al.* (1953) further pointed out that the concentrations of the organophosphorus compounds required to inhibit the growth of the mycobacteria corresponded closely with the concentrations which inhibit the ali-esterases of these bacilli.

Further investigations showed that the growth of certain strains of saprophytic mycobacteria was also very sensitive to inhibition by the organophosphorus compounds (Ruys, unpublished results), whereas the growth of other strains was scarcely affected. This observation offered an opportunity to investigate the suggested correlation between the effects of these inhibitors on the growth and on the ali-esterases of the mycobacteria. In the course of these investigations, it became apparent that some of the mycobacteria must contain more than one ali-esterase. The substrate specificities and other characteristics of these enzymes were therefore studied with the aid of selective inhibitors as described in previous investigations (cf. Myers, 1956).

EXPERIMENTAL

Substrates and inhibitors. The substrates included the esters used in previous investigations (Myers, Schotte, Boer & Borsje-Bakker, 1955; Myers, 1956) together with ethyl phenylacetate (Wislicenus, 1897), acetanilide and butyranilide (Gerhardt, 1853).

The inhibitors used were diethyl *p*-nitrophenyl phosphate (E600, paraoxon), di*iso*propyl *p*-nitrophenyl phosphate (DINP), and phenyl *N*-*n*-propylcarbamate. E600 and DINP were obtained through the courtesy of Albright and Wilson Ltd., London. Phenyl *N*-*n*-propylcarbamate (m.p. 53–54° after recrystallization from *n*-hexane) was synthesized from phenol and *n*-propyl *iso*cyanate by the general method of Kobezen, Metcalf & Fuhuto (1954); the *iso*cyanate derivative was prepared by the procedure described by Schroeter (1909).

Determination of esterase activity. Esterase activity was measured by a manometric method in the Warburg apparatus at 37·5° and pH 7·4 in a medium containing 0·025 M-$NaHCO_3$ and saturated with $CO_2 + N_2$ (5:95, v/v). Preliminary experiments at 25° and 37·5° indicated that the latter temperature was suitable for measuring the esterase activities of the saprophytic mycobacteria as well as those of mammalian tissues. The amount of enzyme preparation was usually adjusted so that the activity was in the range of 2–8 μl. of CO_2/min. in each experiment. The reaction mixture was incubated for 30 min. at 37·5° with or without inhibitor before adding a small volume of substrate, in order to determine the residual esterase activity. All esters were prepared in the form of an aqueous emulsion, suspension or solution containing 5% (w/v) of gum acacia; the final concentrations of ester and gum acacia in the reaction mixture were 0·2 and 0·1% (w/v) respectively. The esterase activity was calculated from the initial constant rate of hydrolysis of the substrate after the appropriate corrections had been made for slow spontaneous hydrolysis.

The rate of hydrolysis of butyranilide and acetanilide was also measured by the manometric technique described above (cf. Michel, Bernheim & Bernheim, 1937); in this case, the concentration of substrate was increased to 1% (w/v). Although the activities were measurable, the data obtained with these substrates are complicated by the relative insolubility of the anilides in water. Butyranilide appeared to be less soluble than acetanilide; this is probably responsible for the fact that butyranilide appeared to be less rapidly hydrolysed than acetanilide by some of the enzymes investigated.

Enzyme preparations. Aqueous homogenates of rat brain and liver were prepared with a glass Potter–Elvehjem homogenizer. As noted previously, the brain suspensions contain several esterases; all of the experiments described here are concerned only with the 'DINP-sensitive' esterase, which is responsible for about 70% of the total activity towards tributyrin and for all of the activity towards ethyl butyrate (Myers, 1956). The liver also contains several enzymes with the characteristics of ali-esterases (Falconer & Taylor, 1946; Fodor, 1946; Myers & Mendel, 1953; Myers, Kemp, Tol & de Jonge, unpublished results); the enzyme studied in the present investigation is responsible for more than 90% of the total activity towards tributyrin and for 99% of the total activity towards ethyl butyrate and phenyl butyrate (cf. Aldridge, 1954). This esterase would seem to be analogous to the ali-esterase from horse liver which was used by Schwert & Glaid (1952).

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The mycobacteria were supplied by Professor A. Charlotte Ruys, Laboratory of Hygiene, University of Amsterdam. The results obtained in growth experiments with *Mycobacterium phlei* and a similar strain of saprophytic mycobacteria designated as S 3048 have been described previously (Ruys, 1954). Five other strains of saprophytic mycobacteria isolated from human-stomach washings were also selected for study; these strains have been designated as *Myco.* A, B, C, D and E in the present paper. (These strains are numbered as VII, XIV, IX, XII and XV respectively in the collection at the Laboratory of Hygiene.) The hitherto unpublished results obtained by Ruys in preliminary growth experiments with these strains are indicated in Table 1; it is hoped that more extensive results will be published elsewhere.

The ali-esterase content of the saprophytic mycobacteria seemed to be independent of the medium on which they were grown. Almost identical results were obtained with samples of the same strain grown in *bouillon*, the Dubos medium (cf. Dubos & Davis, 1946; Dubos & Middlebrook, 1947), the medium of Proskauer & Beck (1894; cf. Youmans & Karlson, 1947), or on the medium of Löwenstein (1930). The activities of the various strains towards tributyrin varied from 50 to 250 μl. of CO_2/mg. dry wt. of bacteria/hr., but the values obtained were found to be characteristic of the strain in question rather than of the culture medium. The sensitivity of the ali-esterases to inhibition by E 600 under the experimental conditions was also independent of the medium on which the bacteria had been grown.

The synthetic medium of Proskauer & Beck (1894) appeared to be the most convenient for culturing large amounts of mycobacteria; most of the experiments on the ali-esterases were carried out with mycobacteria which had been grown on this medium. The mycobacteria were harvested after they had been grown for 4 weeks at 37°; they were centrifuged down from the culture medium at 3000 rev./min. in a clinical centrifuge, washed with boiled water to remove the last traces of the culture medium together with any extracellular proteins, and resuspended in water with the aid of a Potter–Elvehjem homogenizer. The bacterial suspensions were stored at 0° until used. All experiments reported in this paper were carried out within 4 days after preparation of the bacterial suspension; however, the activity of the ali-esterases did not deteriorate appreciably within 2 weeks under these conditions.

RESULTS

The initial experiments on the inhibition of bacterial growth by organophosphorus compounds were carried out with *Mycobacterium tuberculosis* typus *humanus* H 37 Rv; the growth in the Dubos medium at 37° was delayed for several days in the presence of 10^{-5} M E 600 (Mendel *et al.* 1953; Ruys, 1954). DINP, the *iso*propyl analogue of E 600, is considerably more active than E 600 in preventing the growth of the tubercle bacilli (Ruys, unpublished results; see Table 1); this is presumably due to the greater stability of DINP in the Dubos medium. E 600, in contrast to DINP, is slowly broken down by the A-esterase present in the human-serum albumin which is a component of the Dubos medium (Ruys, 1954; cf. Aldridge, 1953).

Mendel *et al.* (1953) showed that the ali-esterase of the tubercle bacilli is strongly inhibited by E 600; however, accurate measurements of the esterase activity with the Warburg apparatus were not carried out owing to the high pathogenicity of these bacilli. Two strains of non-pathogenic mycobacteria, *Myco. phlei* and S 3048, were therefore investigated by Ruys (1954; unpublished results). Although the ali-esterases of both of these strains were almost completely inhibited by 10^{-5} M E 600 within 30 min. at 37° in the Warburg apparatus, the rate of growth in the Dubos medium at 37° was not significantly affected by the same concentration of E 600. It seemed possible that this finding might be explained by the rapid growth of these bacilli at 37°, since other data indicated that the concentration of E 600 required to prevent the growth increases in

Table 1. *Effect of E 600 and DINP on the growth of several strains of mycobacteria*

Mycobacteria were grown in the Dubos medium at 37° by the methods described by Mendel *et al.* (1953) and Ruys (1954). The relative rate of growth was estimated from the time taken to reach a visible turbidity due to the multiplication of the mycobacteria in the medium. This time does not provide an accurate criterion for comparing the rate of growth of different strains of mycobacteria, but does clearly demonstrate the effects of E 600 and DINP when the rate of growth is compared with the control experiments which were carried out simultaneously with the same inoculum of a given strain of mycobacteria. The preliminary results given in this table are taken from unpublished studies of Ruys.

Strain of mycobacteria	Relative rate of growth (days to reach a visible turbidity in the Dubos medium)		
	Control	In the presence of 10^{-5} M E 600	In the presence of 3×10^{-6} M DINP
Myco. tuberculosis H 37 Rv	3	16	(>85)
Myco. D	2½	(>40)	(>85)
Myco. E	3	(>85)	(>85)
Myco. C	4	(>50)	(>50)
Myco. phlei	1	1	—
Myco. A	3	4	3
Myco. B	2	3	2

proportion to the rate of growth (Mendel *et al.* 1953; Ruys, 1954). To test this possibility, five other strains of saprophytic mycobacteria were studied by Ruys (unpublished results). Her results showed that the growth of three of the five strains was strongly inhibited at 37° in the Dubos medium by 10^{-5}M E600 or 3×10^{-6}M DINP; however, the growth of the other two strains was not affected by DINP and only slightly affected by E600 (see Table 1). It seemed unlikely that these differences could be explained by the differences in the rate of growth. The ali-esterases of these saprophytic mycobacteria were therefore studied in detail to see if there was any correlation between the effect of the organophosphorus compounds on the growth and on the esterases of the mycobacteria.

Differentiation of the ali-esterases

In the course of the investigations on the inhibition of the esterases by various compounds, it became apparent that some of the mycobacteria must contain more than one ali-esterase with a measurable activity. The evidence for this conclusion in *Myco. phlei* and *Myco.* B is shown in Table 2; the results obtained with the strain designated as *Myco.* E are also included in this table for purposes of comparison.

With *Myco.* E the degree of inhibition of the ali-esterase activity increases progressively as the concentration of inhibitor is increased until the esterase activity appears to be completely inhibited (Table 2). These results fail to give any indication for the presence of more than one esterase in *Myco.* E which is capable of hydrolysing ethyl butyrate at a measurable rate. The same type of result was obtained with *Myco.* C and *Myco.* D.

With *Myco. phlei*, on the other hand, the inhibition of the esterase activity towards ethyl butyrate reaches a plateau when the concentration of DINP reaches 10^{-6}M; a further increase in the concentration up to 10^{-5}M DINP has little effect on the residual esterase activity (Table 2 and Fig. 1). The existence of this plateau value strongly suggests that *Myco. phlei* must contain at least two ali-esterases capable of hydrolysing ethyl butyrate, one of which is almost completely inhibited by 10^{-6}M DINP, whereas the other is not significantly inhibited before the concentration of DINP is increased above 10^{-5}M. A similar plateau value does not appear clearly when phenyl *N*-*n*-propylcarbamate is used as inhibitor, although the curve relating the esterase activity to the inhibitor concentration is somewhat flattened in the region of 10^{-6}M inhibitor (Fig. 1). However, a marked difference in the sensitivity to this carbamate does become evident when the DINP-resistant esterase activity is measured separately, in the presence of 10^{-5}M DINP to eliminate the activity of the DINP-sensitive esterase. The DINP-resistant activity is almost completely inhibited by 10^{-6}M phenyl *N*-*n*-propylcarbamate, although the total activity in the absence of DINP is only slightly affected by the same concentration of this compound (Table 2 and Fig. 1). It is probable, therefore, that the DINP-resistant activity is due to an esterase which is relatively sensitive to inhibition by the carbamate derivative, whereas the DINP-sensitive activity is due to a second esterase which is less sensitive to the carbamate inhibitor. The DINP-sensitive enzyme, it might be noted, appears to be responsible for the major portion of the total esterase activity towards ethyl butyrate.

Myco. phlei also exhibits a low but measurable activity towards ethyl leucine and butyranilide (Table 3). The results obtained with these substrates confirm the above conclusions. Almost all of the activity of *Myco. phlei* towards ethylleucine is resistant to inhibition by 10^{-6}M phenyl *N*-*n*-propylcarbamate and sensitive to inhibition by 10^{-5}M DINP, whereas the activity towards butyranilide shows the reverse pattern, i.e. resistance to inhibition by 10^{-5}M DINP and sensitivity to 10^{-6}M carbamate (Table 3). The relationship between these results and those obtained with ethyl butyrate can be seen most clearly when the inhibition of the esterase activity is plotted against the inhibitor concentration, as in Fig. 1. The characteristics of the two esterases in *Myco. phlei* are readily apparent from this figure. Most of the activity towards ethylleucine is due to the DINP-sensitive esterase, whereas most of the activity towards butyranilide is due to the DINP-resistant esterase.

The results obtained with *Myco.* B can be analysed in the same way as described above for the esterases of *Myco. phlei*. The evidence again points to the presence of two esterases, but in this case the plateau value representing the borderline between the activities of the two esterases is observed with the inhibitor phenyl *N*-*n*-propylcarbamate and is not distinct with DINP (Table 2). When the activity towards ethyl butyrate is measured in the presence of 10^{-6}M phenyl *N*-*n*-propylcarbamate to eliminate the activity of the carbamate-sensitive esterase, it becomes apparent that the residual carbamate-resistant activity is relatively resistant to inhibition by DINP (Table 2). These results present a contrast to those obtained with *Myco. phlei*; the carbamate-resistant esterase activity of *Myco. phlei* is readily inhibited by DINP.

As with *Myco. phlei*, it is again found that the activities of *Myco.* B towards ethylleucine and butyranilide are, for the greater portion, due to separate enzymes. Almost all of the activity towards ethylleucine is resistant to inhibition by phenyl *N*-*n*-propylcarbamate in concentrations up to 10^{-4}M, whereas almost all of the activity towards

Table 2. *Effect of DINP and phenyl* N-n-*propylcarbamate on the hydrolysis of ethyl butyrate by mycobacteria*

Suspensions of mycobacteria were incubated for 30 min. at pH 7·4 and 37·5° with DINP, phenyl *N-n*-propylcarbamate, or both inhibitors simultaneously, in the concentrations given. Subsequently a small volume of substrate was added and the esterase activity measured manometrically.

Concn. of DINP (−log M)	Concn. of carbamate (−log M)	Esterase activity towards ethyl butyrate (μl. of CO_2/mg. dry wt./hr.)		
		Myco. phlei	*Myco.* B	*Myco.* E
(No inhibitor)		120	75	115
8·0	—	104	75	105
7·0	—	60	73	56
6·0	—	24	59	11
5·0	—	22	28	1
4·0	—	11	9	0
3·0	—	2	2	0
—	9·0	—	74	104
—	8·0	118	69	58
—	7·0	111	45	12
—	6·0	98	19	3
—	5·0	72	15	1
—	4·0	22	14	0
—	3·0	4	7	0
5·0	—	22	—	—
5·0	8·0	20	—	—
5·0	7·0	13	—	—
5·0	6·0	1	—	—
5·0	5·0	0	—	—
—	6·0	98	19	—
8·0	6·0	83	19	—
7·0	6·0	38	19	—
6·0	6·0	2	18	—
5·0	6·0	1	16	—
4·0	6·0	—	8	—
3·0	6·0	—	2	—

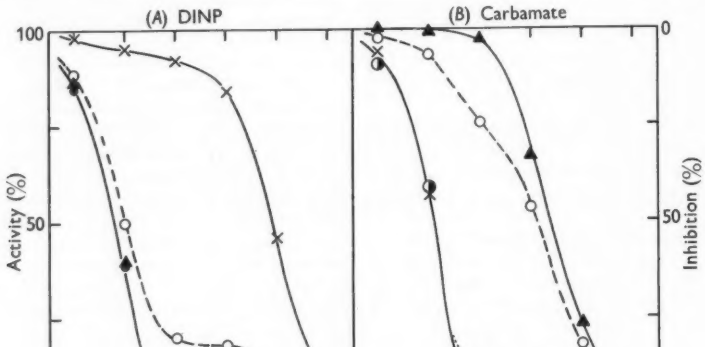

Fig. 1. Effect of DINP and phenyl *N-n*-propylcarbamate on the ali-esterases of *Myco. phlei*. The values are calculated from the data given in Tables 2 and 3. Graph *A* demonstrates the effect of various concentrations of DINP on the total esterase activity towards butyranilide (×), ethylleucine (▲) and ethyl butyrate (○), and on the residual activity towards ethyl butyrate which remains in the presence of 10^{-6} M phenyl *N-n*-propylcarbamate (●). Graph *B* shows the results obtained in parallel experiments with various concentrations of phenyl *N-n*-propylcarbamate; the same symbols are used but (◑) indicates the effect of the carbamate on the residual activity towards ethyl butyrate which remains in the presence of 10^{-5} M DINP (see Table 2).

Table 3. *Effect of DINP and phenyl* N-n-*propylcarbamate on the hydrolysis of ethylleucine and butyranilide by mycobacteria*

Suspensions of mycobacteria were incubated for 30 min. at pH 7·4 and 37·5° with DINP or phenyl *N-n*-propylcarbamate in the concentrations given. Subsequently a small volume of substrate was added and the esterase activity measured manometrically. The amount of mycobacteria used for each measurement was varied between 10 and 100 mg. dry weight depending on the magnitude of the esterase activity in question; the degree of inhibition produced did not appear to be markedly dependent on the amount of enzyme preparation employed.

		Esterase activity (μl. of CO_2/mg. dry wt./hr.)					
		Myco. phlei		*Myco.* B		*Myco.* E	
Concn. of DINP (−log M)	Concn. of carbamate (−log M)	Ethyl-leucine	Butyr-anilide	Ethyl-leucine	Butyr-anilide	Ethyl-leucine	Butyr-anilide
(No inhibitor)		25·5	9·5	1·2	31·0	0·6	4·7
8·0	—	22·1	9·3	1·2	31·0	0·6	4·2
7·0	—	10·1	9·0	1·2	29·0	0·3	2·3
6·0	—	0·5	8·7	1·2	22·5	0·0	0·5
5·0	—	0·4	8·0	1·1	7·7	0·0	0·2
4·0	—	0·2	4·4	0·6	0·2	—	—
3·0	—	0·0	0·8	0·1	0·0	—	—
—	8·0	25·5	8·9	1·2	28·6	0·3	2·4
—	7·0	25·3	5·4	1·2	16·5	0·1	0·5
—	6·0	24·8	1·0	1·2	1·4	0·0	0·2
—	5·0	17·0	0·8	1·1	0·5	—	—
—	4·0	5·8	0·3	1·1	0·4	—	—
—	3·0	0·4	0·0	0·6	0·0	—	—

butyranilide is inhibited by a 100-fold smaller concentration of this compound (Table 3). The results obtained with *Myco.* A were qualitatively identical with those described above for *Myco.* B. With *Myco.* A and *Myco.* B as well as with *Myco. phlei*, therefore, a carbamate-sensitive enzyme is responsible for most of the activity towards butyranilide and a carbamate-resistant enzyme is responsible for most of the activity towards the ethyl ester of leucine. Although DINP rather than phenyl *N-n*-propylcarbamate was used to distinguish between the two esterases of *Myco. phlei* (Table 2), these enzymes will be referred to as the carbamate-sensitive and carbamate-resistant esterases to stress the analogy with the esterases of *Myco.* A and *Myco.* B.

It might be noted that the activity towards butyranilide is probably not due completely to the carbamate-sensitive esterase; conversely, the carbamate-sensitive esterase may also be able to hydrolyse the ethyl ester of leucine slowly. The results given for *Myco. phlei* in Table 3 and Fig. 1 indicate that the carbamate-resistant esterase is probably responsible for about 10% of the total activity towards butyranilide; the results obtained with ethylleucine are less decisive but possibly the carbamate-sensitive esterase may be responsible for about 2% of the total activity of *Myco. phlei* towards this ester. Similarly, the carbamate-resistant esterase of *Myco.* B is probably responsible for about 1·5% of the total activity towards butyranilide (Table 3).

The esterase present in *Myco.* E can also be classified as a carbamate-sensitive enzyme (see Tables 2 and 3). This esterase appears to be responsible both for the very small activity of *Myco.* E towards ethylleucine and for the larger activity towards butyranilide (Table 3). The results obtained with *Myco.* C and *Myco.* D were again almost identical with those shown for *Myco.* E in Table 3.

The characteristics of the esterases described above are summarized in Table 4. This table shows the relative sensitivities of the esterases to inhibition by DINP and by phenyl *N-n*-propylcarbamate, together with the approximate pI_{50} values (negative logarithm of the molar concentration of inhibitor required to produce 50% inhibition of the esterase activity); the pI_{50} values were calculated from results similar to those given in Tables 2 and 3 by a graphical plot of the type shown in Fig. 1. The results obtained with E 600 in other experiments are also included in Table 4 for purposes of comparison.

The ali-esterases of the strains C, D and E exhibit similar characteristics with E 600 as well as with the other two inhibitors (Table 4). However, the remainder of the esterases described here fail to show any marked resemblances in their sensitivities to these inhibitors. For comparative purposes, we also studied the DINP-sensitive esterase of rat brain (cf. Myers, 1956) under the same experimental conditions, as well as the ali-esterase of rat liver which is responsible for the hydrolysis of ethyl and phenyl butyrates by this tissue (cf. Aldridge, 1954); the results shown in Table 4 do not provide any evidence of similarities between these enzymes.

Table 4. *Comparison of various ali-esterases in their sensitivity to three inhibitors*

Enzyme suspensions were incubated for 30 min. at pH 7·4 and 37·5° with the inhibitors before measuring the residual esterase activity; the type of result obtained is illustrated in Tables 2 and 3. The relative inhibition of the various esterases by a critical concentration of inhibitor is designated as complete or almost complete (+ +), partial (+), and slight or insignificant (0) inhibition. The pI_{50} values (−log. of the molar concentration of inhibitor required to produce 50% inhibition of the esterase activity) were estimated from the graphs shown in Fig. 1 and from similar plots of the results which were obtained with the other esterases indicated below. The values for the DINP-sensitive ali-esterase of rat brain were determined with 100 mg. fresh wt. of brain/5 ml. of reaction mixture, with tributyrin as substrate (cf. Myers, 1956). The pI_{50} values for the ali-esterase of rat liver are highly dependent on the concentration of enzyme; the values given below were determined with phenyl butyrate as substrate with 0·4 mg. fresh wt. of liver/5 ml. of reaction mixture.

Esterase		Relative inhibition by			pI_{50} values		
Source	Classification	10^{-6}M phenyl *N*-*n*-propyl-carbamate	10^{-5}M DINP	10^{-5}M E600	Phenyl *N*-*n*-propyl-carbamate	DINP	E600
Myco. C	Carbamate-sensitive	+ +	+ +	+ +	7·9	6·4	6·8
Myco. D	Carbamate-sensitive	+ +	+ +	+ +	8·1	6·1	7·0
Myco. E	Carbamate-sensitive	+ +	+ +	+ +	8·0	6·2	7·0
Myco. phlei	Carbamate-sensitive	+ +	0	+	6·8	4·0	5·1
Myco. phlei	Carbamate-resistant	0	+ +	+ +	4·5	7·2	7·8
Myco. A	Carbamate-sensitive	+ +	+	+	7·6	4·8	5·2
Myco. A	Carbamate-resistant	0	0	+	3·3	3·9	5·0
Myco. B	Carbamate-sensitive	+ +	+	+ +	7·0	5·5	6·8
Myco. B	Carbamate-resistant	0	0	+ +	3·1	4·0	6·1
Rat brain	DINP-sensitive	0	+ +	+ +	2·6	7·3	7·4
Rat liver	DINP-sensitive	+	+ +	+ +	5·7	8·9	9·0

Substrate specificity of the ali-esterases

On the basis of the differences between the esterases in their sensitivity to DINP and phenyl *N*-*n*-propylcarbamate (Table 4), it is possible to distinguish the activities of each of these esterases and consequently to study their substrate specificities. The results obtained in this way are shown in Table 5. To facilitate comparison of these results the esterases have been grouped according to their sensitivity to inhibition by phenyl *N*-*n*-propylcarbamate. The brain esterase could also be classified in the group of carbamate-resistant esterases (Table 4), whereas the ali-esterase of liver appears to occupy an intermediate position.

The results given in Table 5 show that the carbamate-sensitive esterases of *Myco.* C, D and E are not completely identical although they are very similar; on the basis of the results given in Tables 4 and 5, it does seem justified to consider these esterases as variations of one distinct enzyme. The carbamate-sensitive esterases of *Myco. phlei* and strains A and B also show some similarity to each other and to the esterases of strains C, D and E (Table 5), even though they differ considerably in their sensitivities to inhibition by E600 and DINP (Table 4). The most striking differences in the relative substrate-specificity patterns are observed with the three phenyl-substituted esters; the rate of hydrolysis by the carbamate-sensitive esterases *Myco. phlei* and strains A and B decreases in the order ethyl β-phenylpropionate, ethyl phenylacetate, ethyl benzoate, whereas the rate of hydrolysis by the carbamate-sensitive esterases of strains C, D and E decreases in the reverse order, i.e. ethyl benzoate, ethyl phenylacetate, ethyl β-phenylpropionate.

The two carbamate-resistant esterases of *Myco. phlei* and *Myco.* B also show some resemblance to each other and to the ali-esterase of rat liver, although the liver esterase appears to hydrolyse all of the ethyl esters more efficiently than do the bacterial esterases in question (Table 5). In contrast to the carbamate-sensitive esterases, all of these enzymes exhibit an appreciable activity towards ethylleucine and little activity towards butyranilide. The DINP-sensitive esterase of rat brain failed to exhibit an appreciable activity towards either of these substrates (Table 5), and would therefore not seem to be related to any of the esterases found in the saprophytic mycobacteria with the possible exception of the carbamate-resistant esterase of *Myco.* A. This enzyme also exhibits an exceptional specificity pattern; it does not hydrolyse ethyl esters at an appreciable rate under the experimental conditions (Table 5.)

DISCUSSION

The present investigation was designed primarily to test the possible correlation between the effects of the organophosphorus compounds on the growth and on the ali-esterases of saprophytic mycobacteria. The classification of these ali-esterases was found to be more complicated than expected. However, the results show two general differences between the

ali-esterases of *Myco. phlei* and strains A and B, on the one hand, and the ali-esterases of strains C, D and E on the other hand.

(1) Two distinct esterases can be identified in the bacilli whose growth is relatively resistant to inhibition by E 600 and DINP, i.e. *Myco. phlei* and strains A and B. On the other hand, only one esterase could be identified in the bacilli whose growth in the Dubos medium is readily inhibited by E 600 and DINP, i.e. strains C, D and E. The significance of this finding as an explanation for the effects of E 600 and DINP on the growth is open to some question. In the first place, it seems likely that the strains C, D and E might also contain other esterases but that the amounts would be too small to be distinguished with the substrates and

Table 5. *Comparison of the relative substrate specificities of the various ali-esterases*

Activity of the ali-esterases towards tributyrin is expressed as μl. of CO_2/mg. dry wt./hr., and the relative activity towards the other substrates is calculated as a percentage of the activity towards tributyrin. The values in parentheses were too low to be determined accurately; omission of a value indicates that the activity was not measured. In one case with *Myco.* A and ethylleucine, it was not possible to obtain a reproducible value with different enzyme preparations. Most of the results on the relative substrate specificity of the DINP-sensitive, carbamate-resistant esterase of rat brain are taken from Myers (1956). The activities of each of these esterases were distinguished on the basis of their sensitivities to inhibition by DINP and phenyl *N*-*n*-propylcarbamate as indicated in Table 4.

	Activity of the carbamate-sensitive esterases					
Substrate	*Myco. phlei*	*Myco.* A	*Myco.* B	*Myco.* C	*Myco.* D	*Myco.* E
	(μl. of CO_2/mg. dry wt./hr.)					
Tributyrin	20	35	120	95	48	48
	Relative activity (%)					
Tributyrin	100	100	100	100	100	100
Tripropionin	85	145	55	205	220	265
Triacetin	45	25	15	46	54	65
Phenyl butyrate	175	180	140	230	220	255
Phenyl propionate	150	225	95	310	310	390
Phenyl acetate	105	50	45	155	200	220
Ethyl butyrate	110	150	75	190	240	180
Ethyl β-phenylpropionate	45	60	10	35	58	35
Ethyl phenylacetate	20	40	8	51	65	50
Ethyl benzoate	(10)	20	(2)	62	75	60
Ethylleucine	(2)	(0)	(0)	1	1	1
Ethyl-β-phenylalanine	(0)	(0)	(0)	(0)	(0)	(0)
Butyranilide	40	10	26	9	12	10
Acetanilide	50	—	—	—	8	6

	Activity of the carbamate-resistant esterases			Activity of the DINP-sensitive esterases	
Substrate	*Myco. phlei*	*Myco.* A	*Myco.* B	Rat liver	Rat brain
	(μl. of CO_2/mg. dry wt./hr.)				
Tributyrin	240	16	29	820	22
	Relative activity (%)				
Tributyrin	100	100	100	100	100
Tripropionin	90	90	105	120	165
Triacetin	35	65	30	20	23
Phenyl butyrate	205	240	400	340	225
Phenyl propionate	190	205	420	355	275
Phenyl acetate	60	160	125	140	195
Ethyl butyrate	40	(5)	50	330	10
Ethyl β-phenylpropionate	32	10	70	240	—
Ethyl phenylacetate	10	(0)	18	85	—
Ethyl benzoate	7	(0)	(1)	25	—
Ethylleucine	10	(?)	4	34	(0)
Ethyl-β-phenylalanine	1·5	(0)	3	13	(0)
Butyranilide	0·4	(0)	(1)	0·4	(>1)
Acetanilide	1	—	—	0·7	—

experimental methods used. Secondly, the carbamate resistant esterase of *Myco. phlei* is readily inhibited by E 600 and DINP; the carbamate-resistant esterase of *Myco.* B is also fairly sensitive to inhibition by E 600 (Table 4). This fact suggests that the presence of a carbamate-resistant esterase in these two strains of saprophytic mycobacteria could not explain the lack of effect of E 600 on their growth.

(2) The carbamate-sensitive esterases of *Myco. phlei* and the strains A and B act most efficiently on esters which have a larger acyl group than the esters which are hydrolysed most efficiently by the carbamate-sensitive esterases of the strains C, D and E. This difference is seen most clearly in the relative rates of hydrolysis of the three phenyl-substituted esters, as mentioned previously. It is possible, therefore, that the first group of esterases normally act on a substrate which has a larger acyl residue than the physiological substrate of the second group of esterases. The significance of this difference for the problem in question is uncertain; the remainder of the substrate-specificity pattern (Table 5) gives no indication that the general type of physiological substrate is essentially different.

The results of these experiments do not disprove the hypothesis that the effect of E 600 and DINP on the growth of the mycobacteria is related to the effect of these inhibitors on the ali-esterase activity (cf. Mendel *et al.* 1953). If we compare the esterase activities of the mycobacteria whose growth is resistant to E 600 and DINP with the esterase activities of the mycobacteria whose growth is readily prevented by these compounds under the conditions given in Table 1, we see that the general pattern of ali-esterase activity is the same in the members of each group, although differing from the general pattern of ali-esterase activity in the other group. This fact does suggest that the hypothesis may be correct.

It is not difficult to understand why the growth of the strains C, D and E could be prevented by the esterase inhibitors since the ali-esterases are readily inhibited by E 600 and DINP. Moreover, the esterases of these mycobacteria hydrolyse butyranilide and acetanilide fairly efficiently (Table 5); this finding suggests that these enzymes have a function in protein metabolism, i.e. in the hydrolysis of peptide or amide derivatives or both (cf. Michel *et al.* 1937). Under these circumstances it would not be surprising that these enzymes should prove to be essential for the normal growth of the mycobacteria.

However, a problem remains in connexion with the fact that E 600 and DINP have little effect on the growth of *Myco. phlei* and the strains A and B, even though certain of the ali-esterases concerned may be inhibited by these compounds. As indicated above, the presence of a second type of enzyme, the carbamate-resistant esterases, in these strains of saprophytic mycobacteria does not seem to provide a sufficient explanation of this problem. In *Myco.* B both the carbamate-sensitive and the carbamate-resistant esterases are fairly sensitive to inhibition by E 600 (Table 4); both enzymes are almost completely inhibited by 10^{-5} M E 600 within 30 min. at 37·5°, although this concentration of inhibitor has little effect on the growth of *Myco.* B (Table 1). A similar situation is encountered with *Myco. phlei.* The carbamate-sensitive esterase is less readily inhibited by E 600 in this case; however, the carbamate-resistant esterase, which is responsible for the major portion of the activity towards tributyrin, ethyl butyrate and most of the other esters tested (Table 2 and 5), is highly sensitive to inhibition by E 600 and DINP (Table 4) and does not seem to be necessary for the growth of these bacilli (cf. Table 1). The results obtained with *Myco.* A are less significant since both esterases are moderately resistant to inhibition by E 600 and DINP (Table 4), and since the carbamate-resistant esterase exhibits a different substrate specificity (Table 5).

We might comment on the similarity in the substrate-specificity patterns of the ali-esterase of rat liver and the carbamate-resistant esterases found in *Myco. phlei* and *Myco.* B. The physiological significance of the liver esterase is also obscure; this enzyme does not seem to be of any immediate importance either for the oxidative metabolism of liver slices or for the regeneration of the liver after partial hepatectomy (cf. Myers & Mendel, 1953). Although we have little further information concerning the hydrolytic enzymes present in the mycobacteria, more extensive experiments have been carried out with liver preparations. Since the ali-esterase in liver seems to resemble the carbamate-resistant esterases in *Myco. phlei* and *Myco.* B, with regard both to their substrate specificities (Table 5) (cf. Cohen, Kushnick & Purdy, 1953) and their apparent dispensability for growth processes, it may be appropriate to discuss the enzymes in liver in more detail at this point.

Despite the fact that the ali-esterase of liver hydrolyses butyranilide fairly slowly (Table 5) and does not seem to exhibit any appreciable activity towards *N*-ethylbutyramide (Schwert & Glaid, 1952), we would suggest that this enzyme may have a function in protein metabolism. The results obtained in other experiments with selective esterase inhibitors indicate that the liver esterase does hydrolyse butyramide slowly (Myers, Kemp, Tol & de Jonge, unpublished results) and that it is probably identical with the liver amidase described by Bray, James, Thorpe & Wasdell (1950) and Bray, White & Wood (1955). In both cases, the enzyme activity shows an optimum between pH 8 and 9, and ex-

hibits the phenomenon of excess-substrate inhibition (Bamann, 1929, 1931; Murray & King, 1930; Bray *et al.* 1955). The activity of the amidase shows the following pattern, in decreasing order: caproamide, butyramide, acetamide, and γ-phenylbutyramide, β-phenylpropionamide, phenylacetamide, benzamide; this specificity pattern agrees well with the results reported above (Table 5), and by Sobotka & Glick (1934) for the esterase activity of liver. The amidase appears to be present in the same fraction of the liver as the enzyme which hydrolyses β-phenylalaninamide and leucinamide (Meister, Levintow, Greenfield & Abendschein, 1955). The similarity in the structure of these substrates suggests that the amidase activities towards β-phenylalaninamide and β-phenylpropionamide might well be due to the same enzyme; further experiments will be required to test the validity of this hypothesis.

The experimental results reported in the literature indicate that the liver contains at least two enzymes capable of hydrolysing leucinamide and β-phenylalaninamide. One of these enzymes, which is probably similar to the leucine aminopeptidase isolated from other tissues, is most active at a slightly acid pH, is strongly activated by manganese ions, does not hydrolyse amino acid esters and is not inhibited by organophosphorus compounds (cf. Smith, 1951*a*, *b*; Smith & Spackman, 1955). The other enzyme is active at more alkaline pH values and does not seem to require activating ions (cf. Errera & Greenstein, 1948; Meister *et al.* 1955). If, as we suggest, the latter enzyme is identical with the ali-esterase of liver, then it would be capable of hydrolysing esters as well as amide derivatives and would be sensitive to inhibition by organophosphorus compounds; similar properties have been demonstrated for other proteolytic enzymes such as trypsin and chymotrypsin (Neurath & Schwert, 1950).

Under these circumstances it is conceivable that complete inhibition of the liver ali-esterase by organophosphorus compounds might not have any obvious influence on the metabolism of this tissue, since the liver also contains another enzyme which is capable of hydrolysing the amino acid amides and which is not affected by the organophosphorus compounds. This seems at present to be the most logical explanation of the results mentioned above which we obtained previously in an investigation of the liver ali-esterase (Myers & Mendel, 1953). It is possible that an analogous situation might explain the results reported here for *Myco. phlei* and *Myco.* B.

The peptidases of mycobacteria have been studied by Roulet & Zeller (1945, 1948), but the connexion between the enzymes which they described and the ali-esterases described above is still a matter for speculation. If, as we suggest, the carbamate-resistant and the carbamate-sensitive esterases of the mycobacteria may both be concerned with the hydrolysis of amides or peptides or both, a further study of the activity of these enzymes towards amino acid derivatives would be valuable in elucidating their physiological functions. It is to be expected that these investigations would shed further light on the reason for the differences observed in the effects of E 600 and DINP on the growth of these mycobacteria.

SUMMARY

1. It had been observed that the growth of certain strains of saprophytic mycobacteria was prevented by organophosphorus compounds in low concentrations, whereas the growth of other strains of saprophytic mycobacteria was not affected under the same experimental conditions (Ruys, unpublished results). This observation provided an opportunity to investigate the suggested correlation between the effects of the organophosphorus compounds on the growth and on the esterase activities of the mycobacteria (Mendel *et al.* 1953).

2. Studies on the substrate specificities and sensitivities of these enzymes to inhibitors showed distinct differences between the ali-esterases of the two groups of mycobacteria. The significance of these differences is discussed.

3. The ali-esterases of the mycobacteria, like the ali-esterase of liver, are capable of hydrolysing a wide variety of esters, including amino acid esters as well as certain amides. It is suggested therefore that these enzymes might have a function in protein metabolism. On this basis it is possible to offer an explanation for the differences in the effects of the organosphosphorus compounds on growth.

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Studies on Ali-esterases

6. SELECTIVE INHIBITORS OF THE ESTERASES OF BRAIN AND SAPROPHYTIC MYCOBACTERIA*

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Previous investigations have shown that the growth of *Mycobacterium tuberculosis* and certain strains of saprophytic mycobacteria can be prevented *in vitro* by certain organophosphorus compounds (Mendel, Myers, Uyldert, Ruys & de Bruyn, 1953; Ruys, 1954; Myers, Tol & de Jonge, 1957); it was suggested that the effect of these compounds on growth may be related to their ability to inhibit the ali-esterases of these mycobacteria. The organophosphorus compounds which were used in these experiments cannot be used for the same purpose *in vivo* since they are also potent inhibitors of true cholinesterase and are consequently highly toxic to animals. In an attempt to find more selective inhibitors of the ali-esterases, a series of compounds was synthesized and tested initially against the true cholinesterase and ali-esterase of rat brain. Some of the results obtained in these preliminary experiments have been reported previously (Myers, Mendel, Gersmann & Ketelaar, 1952; Mendel *et al.* 1953; Myers, 1953, 1954; Myers & Kemp, 1954).

In the present work a number of new inhibitory compounds were prepared and investigated as inhibitors of the ali-esterases of the saprophytic mycobacteria and of the esterases of rat brain *in vitro*. The results obtained appear to offer valuable information concerning the factors which govern the selectivity of these inhibitors towards a given esterase.

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EXPERIMENTAL

Determination of esterase activity. Esterase activity was measured by a manometric method in the Warburg apparatus at 37·5° and pH 7·4 in a medium containing 0·025 M-$NaHCO_3$ and saturated with $CO_2 + N_2$ (5:95, v/v). The reaction mixture was incubated for 30 min. at 37·5°, with or without added inhibitor, before adding a small volume of substrate to determine the residual esterase activity. The ali-esterase activities were measured with 0·2% (w/v) of tributyrin as substrate and the true cholinesterase activity of rat brain with 0·03 M acetyl-β-methylcholine. All values were corrected for the very slow spontaneous hydrolysis of the substrate in question under the same experimental conditions.

The enzyme preparations used were described in previous publications (Myers, 1956*b*; Myers *et al.* 1957); for comparative purposes, we have tested the true cholinesterase and ali-esterase of rat-brain homogenates, and the ali-esterases of five strains of saprophytic mycobacteria. Two of these strains, *Mycobacterium phlei* and *Myco.* B, are not affected by the organophosphorus compounds in their growth at 37° in the medium of Dubos & Davis (1946), even though the ali-esterase activity is inhibited (Myers *et al.* 1957); the growth of the other three strains, which were

designated as *Myco.* C, D and E, is readily inhibited under the same conditions (Myers *et al.* 1957). It might be noted that the hydrolysis of tributyrin by *Myco.* C, D and E seems to be due to a single esterase and the hydrolysis of acetyl-β-methylcholine by rat-brain suspensions is, of course, also due to a single enzyme, the true cholinesterase. In the other cases, it was possible to distinguish more than one esterase in the preparation capable of hydrolysing tributyrin by the use of di*iso*propyl *p*-nitrophenyl phosphate (DINP) and phenyl *N*-*n*-propylcarbamate as selective inhibitors (Myers, 1956*b*; Myers *et al.* 1957). For purposes of convenience in the execution of the experiments, we have only considered the esterase in each preparation which is responsible for the major portion of the activity towards tributyrin. Thus the experiments reported below are concerned with the 'DINP-sensitive' esterase of rat brain (cf. Myers, 1956*b*), the 'carbamate-resistant' or 'DINP-sensitive' esterase of *Myco. phlei*, and the 'carbamate sensitive' esterase of *Myco.* B; these esterases are responsible for approximately 70, 92 and 80% respectively of the total activity of each preparation towards tributyrin.

Under these circumstances, the molar concentration of inhibitor required to produce 50% inhibition of the esterase (I_{50}) could be readily estimated from the graphs relating the concentration of inhibitor to the percentage inhibition of the esterase activity (cf. Myers *et al.* 1957). The results were expressed as the negative logarithm of the I_{50} value (pI_{50}). The pI_{50} value was found to be reproducible to within 0·1 unit in duplicate experiments carried out at different times over a period of several months.

Inhibitors. The inhibitors used in these experiments are listed in Table 1. Compounds 1 (tetraethyl pyrophosphate, TEPP), 6 and 18 were supplied by Dr H. R. Gersmann, Laboratory of General and Inorganic Chemistry, University of Amsterdam. Compound 2 (di*iso*propyl phosphorofluoridate, DFP) was obtained from Dr B. C. Saunders, Cambridge University, England; compound 3 (Mipafox, Isopestox) from Dr G. S. Hartley, Pest Control Ltd., England; compounds 9 (diethyl *p*-nitrophenyl phosphate, E600, Paraoxon) and 10 (di*iso*propyl *p*-nitrophenyl phosphate, DINP) from Mr B. Topley, Albright and Wilson Ltd., England; compounds 12 (Ro3-0620) and 29 (Ro3-0717) from Dr A. L. Morrison, Roche Products Ltd., Welwyn Garden City, England; compound 37 (*NN*-diethylcarbamoyl fluoride) from Dr G. Schrader, Elberfeld, Germany; and compound 49 (Ro2-3510) from Dr J. A. Aeschlimann, Hoffmann-La Roche Inc., Nutley, N.J., U.S.A. Most of these compounds are described in Kosolapoff (1950) and Schrader (1952).

The remaining compounds were synthesized in this laboratory; the melting points are uncorrected. Compounds 4 (m.p. 144°) and 5 (m.p. 168–170°) are described by Cook *et al.* (1949) and were prepared by the same methods. Compound 8 was prepared by the method described by Cebrian (1951) and compound 7 (m.p. 81–83°) was prepared in the same way with *p*-nitrophenyl phosphorodichloridate and methylamine. Compound 11 (m.p. 154–156°) is described by Ketelaar & Gersmann (1950).

The following compounds were prepared from sodium *p*-nitrophenoxide and the corresponding phosphoryl chloride derivative in dioxan, by the techniques described by Ketelaar & Gersmann (1950) for the synthesis and purification of compounds 11 and 18; the phosphoryl chloride derivatives are described in the literature cited below. Compound 13 (m.p. 117–118°) was prepared from sodium *p*-nitrophenoxide and *ON*-diphenyl phosphoramidochloridate (Michaelis, 1903); compound 14 (m.p. 178–179°) from the chloride listed as compound 5 (Cook *et al.* 1949); compound 15 (m.p. 113–114°) from phenyl phosphorodichloridate (Freeman & Colver, 1938); compound 16 (m.p. 175–176°) from *N*-phenylphosphoramidic dichloride (Michaelis & Schulze, 1893); compound 17 (m.p. 147°) from benzylphosphonic dichloride (Kinnear & Perren, 1952); compound 20 (m.p. 135–137°) from *N*-methylphosphoramidic dichloride (Michaelis, 1903); compound 21 (m.p. 118°) from chloromethylphosphonic dichloride (Kinnear & Perren, 1952); compound 22 (m.p. 155°) from ethylphosphonic dichloride (Kinnear & Perren, 1952); compound 23 (m.p. 148°) from *NN*-dimethylphosphoramidic dichloride (Michaelis, 1903); compound 24 (m.p. 112°) from αα-dichloromethylphosphonic dichloride (Kinnear & Perren, 1952); compound 25 (m.p. 93–94°) from *iso*propylphosphonic dichloride (Kinnear & Perren, 1952); compound 26 (m.p. 130–131°) from *cyclo*hexylphosphonic dichloride (Graf, 1952); and compound 27 (m.p. 103°) from phenylphosphonic dichloride (Michaelis & Kammerer, 1875). The identity of all of these compounds was confirmed by the constant melting points obtained after successive recrystallizations and by determinations of the amount of *p*-nitrophenol liberated by hydrolysis in alkali.

Compound 19 (m.p. 107°) was prepared from the corresponding phosphorothioate (Ketelaar & Gersmann, 1950) by isomerization at 140° as described by Myers *et al.* (1952); compound 28 was prepared in the same way from the corresponding phosphorothioate which was supplied under the designation E1907 by Dr G. Schrader, Elberfeld, Germany (cf. Mendel *et al.* 1953). Compounds 30 (m.p. 92–93°), 31 (m.p. 93°) and 32 (m.p. 105°) were prepared in dioxan from *N*-methylphosphoramidic dichloride (Michaelis, 1903) with the sodium salts of *p*-chlorophenol, phenol and *p*-cresol respectively. Compound 33 (m.p. 154–155°) was prepared by the method of Autenrieth (1897) and compound 34 (b.p. 250° at 20 mm.) by the method of Michaelis & Kaehne (1898).

The two sulphonyl fluoride derivatives (compounds 35 and 36) were prepared previously (Myers & Kemp, 1954) by the method of Davies & Dick (1932). *N*-Phenylcarbamoyl fluoride (compound 38; m.p. 30–31°) is described by Buckley, Piggot & Welch (1945). Most of the remaining carbamate derivatives (compound 39, m.p. 127–129°; compound 40, m.p. 113–114°; compound 41, m.p. 147–148°; compound 43, m.p. 46–47°; compound 44, m.p. 85–86°; compound 45, m.p. 97°; compound 46, m.p. 116–117°; compound 47, m.p. 88–89°; compound 48, m.p. 157–159°) are described by Kobezen, Metcalf & Fuhuto (1954). Phenyl *N*-*n*-propylcarbamate (compound 42, m.p. 53–54°) was prepared in the same way from phenol and *n*-propyl *iso*cyanate (cf. Myers *et al.* 1957).

Many of these inhibitors are sparingly soluble in water and consequently the stock solutions of inhibitor were prepared with acetone. The final concentration of acetone in the reaction mixture with the enzyme preparations was usually 1% (v/v). In these cases, the activity of the enzyme plus inhibitor was compared with the activity of the uninhibited enzyme in a control vessel containing the same amount of acetone. However, the concentration of acetone used had little or no effect on the esterase activity of the uninhibited enzyme.

Table 1. *Inhibitory activity of various compounds towards the esterases of brain and of mycobacteria*

Determination of the inhibitory activity of these compounds is described in the text. pI_{50} is the negative logarithm of the molar concentration of inhibitor required to produced 50% inhibition of the esterase activity after incubation for 30 min. at 37·5° and pH 7·4. The following abbreviations are used in writing the formulae of the inhibitory compounds: Me-, methyl; Et-, ethyl; Pr-, *n*-propyl; *iso*Pr-, *iso*propyl; Ph-, phenyl; *cyclo*Hx-, *cyclo*hexyl; Ph-CH_2-, benzyl; *p*-Me-Ph-, *p*-cresyl; *p*-Cl-Ph-, *p*-chlorophenyl; *o*-Cl-Ph-, *o*-chlorophenyl; *p*-NO_2-Ph-, *p*-nitrophenyl.

Compound no.	Structural formula	pI_{50} values with the following ali-esterases					pI_{50} value with the true cholinesterase of rat brain
		Rat brain	*Myco. phlei*	*Myco.* B	*Myco.* D	*Myco.* E	
1	$(Et\text{-}O)_2P(O)\text{-}O\text{-}P(O)(O\text{-}Et)_2$	5·9	7·8	4·9	4·8	4·7	8·2
2	$F\text{-}P(O)(O\text{-}iso\text{Pr})_2$	5·1	6·9	4·9	5·5	5·8	6·7
3	$F\text{-}P(O)(NH\text{-}iso\text{Pr})_2$	4·2	6·0	3·8	5·4	5·6	3·5
4	$F\text{-}P(O)(NH\text{-}Ph)_2$	4·3	4·7	4·0	4·9	5·1	4·8
5	$Cl\text{-}P(O)(NH\text{-}Ph)_2$	3·1	3·6	3·4	3·8	3·8	2·4
6	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)Me_2$	5·0	5·3	—	3·0	—	4·4
7	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(NH\text{-}Me)_2$	5·9	6·2	4·8	4·0	4·0	4·2
8	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(O\text{-}Me)_2$	6·6	7·1	5·0	6·1	6·3	7·7
9	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(O\text{-}Et)_2$	7·4	7·8	6·8	7·0	7·0	8·0
10	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(O\text{-}iso\text{Pr})_2$	7·3	7·2	5·5	6·1	6·2	7·3
11	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(O\text{-}Ph\text{-}NO_2\text{-}p)_2$	4·8	6·0	5·0	5·0	5·1	4·4
12	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(O\text{-}Ph)_2$	7·2	6·7	5·3	6·9	7·1	6·3
13	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(NH\text{-}Ph)\text{-}O\text{-}Ph$	6·5	6·1	4·8	5·4	5·7	3·5
14	$p\text{-}NO_2\text{-}Ph\text{-}O\text{-}P(O)(NH\text{-}Ph)_2$	6·4	6·2	5·4	5·6	5·9	3·9
15	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(O)\text{-}O\text{-}Ph$	6·9	6·7	5·3	6·0	6·1	6·0
16	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(O)\text{-}NH\text{-}Ph$	6·4	6·2	5·8	6·0	6·0	4·7
17	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(O)\text{-}CH_2\text{-}Ph$	7·3	6·5	7·1	7·7	7·5	5·1

Table 1 (*cont.*)

Compound no.	Structural formula	pI_{50} values with the following ali-esterases: Rat brain	*Myco. phlei*	*Myco.* B	*Myco.* D	*Myco.* E	pI_{50} value with the true cholinesterase of rat brain
18	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}O\text{-}Me$	6·9	7·6	6·9	5·3	—	7·4
19	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}S\text{-}Me$	6·5	7·3	6·8	5·3	5·1	4·9
20	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}NH\text{-}Me$	7·7	7·2	6·9	5·6	5·4	5·3
21	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}CH_2\text{-}Cl$	7·4	7·2	6·6	5·7	5·5	5·1
22	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}Et$	7·5	7·8	6·7	5·8	5·7	6·0
23	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}NMe_2$	5·6	6·7	4·9	4·6	4·4	5·0
24	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}CHCl_2$	5·6	6·6	4·9	4·9	4·9	5·1
25	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}iso\text{Pr}$	5·7	6·9	4·7	4·5	4·4	3·9
26	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}cyclo\text{Hx}$	4·8	7·2	6·2	6·0	6·0	(<3·0)
27	$(p\text{-}NO_2\text{-}Ph\text{-}O)_2P(=O)\text{-}Ph$	5·4	7·7	6·0	5·7	5·7	6·3
28	$(EtO.OC\text{-}Ph\text{-}O)_2P(=O)\text{-}S\text{-}Me$	6·3	7·2	6·5	5·3	5·0	5·0
29	$(MeO.OC\text{-}Ph\text{-}O)_2P(=O)\text{-}NH\text{-}Me$	7·6	6·7	5·2	5·0	4·8	5·4
30	$(p\text{-}Cl\text{-}Ph\text{-}O)_2P(=O)\text{-}NH\text{-}Me$	6·2	6·1	4·0	4·1	4·1	(*ca.* 3)
31	$(Ph\text{-}O)_2P(=O)\text{-}NH\text{-}Me$	5·2	4·5	3·2	2·5	2·6	3·2
32	$(p\text{-}Me\text{-}Ph\text{-}O)_2P(=O)\text{-}NH\text{-}Me$	4·5	3·4	(*ca.* 2·5)	(<2·5)	(<2·5)	(<2·5)
33	$(p\text{-}Cl\text{-}Ph\text{-}O)_2P(=O)\text{-}NH_2$	5·9	6·3	5·4	5·4	5·3	4·6
34	$(p\text{-}Cl\text{-}Ph\text{-}O)_2P(=O)\text{-}Me$	4·3	5·7	4·8	4·7	4·7	3·0
35	$F\text{-}SO_2\text{-}Me$	3·8	3·8	3·6	3·8	4·0	4·9
36	$F\text{-}SO_2\text{-}CH_2\text{-}Cl$	5·3	5·6	6·0	6·3	6·2	5·3
37	$F\text{-}C(=O)\text{-}NEt_2$	4·8	6·0	4·4	5·3	5·5	5·0
38	$F\text{-}C(=O)\text{-}NH\text{-}Ph$	3·1	1·9	2·2	2·9	2·7	3·2

Table 1 (*cont.*)

Compound no.	Structural formula	pI_{50} values with the following ali-esterases: Rat brain	*Myco. phlei*	*Myco.* B	*Myco.* D	*Myco.* E	pI_{50} value with the true cholinesterase of rat brain
39	Ph-O-C(=O)-NH-Ph	2·8	2·7	3·9	5·7	5·6	2·8
40	*p*-Me-Ph-O-C(=O)-NH-Ph	3·4	2·4	3·7	6·0	5·9	3·2
41	*p*-Cl-Ph-O-C(=O)-NH-Ph	3·7	3·8	4·4	5·9	5·8	(<3)
42	Ph-O-C(=O)-NH-Pr	2·6	4·5	7·0	8·1	8·0	(*ca.* 2·3)
43	Ph-O-C(=O)-NH-Et	2·8	3·7	5·8	7·1	7·0	2·8
44	Ph-O-C(=O)-NH-Me	3·1	3·2	4·5	6·0	6·0	4·0
45	*p*-Me-Ph-O-C(=O)-NH-Me	3·5	3·0	4·2	5·8	5·8	4·2
46	*p*-Cl-Ph-O-C(=O)-NH-Me	3·8	3·9	4·7	6·1	6·1	3·9
47	*o*-Cl-Ph-O-C(=O)-NH-Me	3·9	4·0	4·8	6·7	6·7	5·3
48	*p*-NO_2-Ph-O-C(=O)-NH-Me	3·5	3·4	4·2	4·8	4·8	3·4
49	*p*-NO_2-Ph-O-C(=O)-NMe_2	3·9	4·7	4·3	4·8	4·8	3·9

RESULTS

The results obtained are summarized in Table 1. The characteristics of the esterases in question have been described in previous investigations (Myers, 1956*b*; Myers *et al.* 1957); data on the rate of hydrolysis, dipole moments and absorption spectra of many of the organophosphorus compounds are given by Ketelaar, Gersmann & Koopmans (1952) and by Gersmann (1956). Data on the rate of hydrolysis of the carbamate esters are given by Kobezen *et al.* (1954).

Owing to the complicated nature of the esterases present, we have not studied the effects of the inhibitors listed with the strain of saprophytic mycobacteria which was designated as *Myco.* A in the previous investigation (Myers *et al.* 1957). However, it was observed that neither of the esterases present in *Myco.* A was very sensitive to inhibition by compound 26; in this respect, these esterases differ from the esterases of the other mycobacteria shown in Table 1.

Table 1 does not include the results obtained with the strain designated as *Myco.* C. As noted previously, the ali-esterase of *Myco.* C is very similar to the esterases of *Myco.* D and E (Myers *et al.* 1957); this conclusion was confirmed in the present investigation. pI_{50} values were determined with *Myco.* C for the compounds 7, 9, 10, 12, 17, 19, 20, 26, 27, 39, 42, 43 and 44. In no case did the pI_{50} value with *Myco.* C differ by more than 0·3 unit from the average of the pI_{50} values given in Table 1 for *Myco.* D and E. As can be seen from Table 1, the pI_{50} values obtained with *Myco.* D and E are nearly identical in most instances and seldom differ by more than 0·2 unit from each other.

The results given for the DINP-sensitive ali-esterase of rat brain are slightly different from those reported for some of the inhibitors in earlier publications, owing to the fact that the presence of more than one ali-esterase in the brain was not taken into consideration previously. When only that portion of the total activity which is due to the DINP-sensitive esterase (i.e. 70%) is considered, most of the pI_{50} values become slightly greater than those estimated on the basis of the total ali-esterase activity towards tributyrin. However, the necessary corrections in the pI_{50} values do not amount to

more than 0·2–0·3 unit. This correction is somewhat uncertain in all cases since it is based on the shape of the curves obtained when the percentage inhibition of the esterase activity is plotted against the logarithm of the molar concentration of inhibitor. Nevertheless, these differences are of minor significance in comparison with the much greater differences between the pI_{50} values for different types of esterases (see Table 1).

DISCUSSION

The original purpose of the present investigation was to find a compound which would act as a selective inhibitor of the ali-esterases in general without inhibiting the cholinesterases, in the same way that eserine and prostigmine selectively inhibit the cholinesterases without inhibiting the ali-esterases (cf. Easson & Stedman, 1937; Richter & Croft, 1942; Mendel & Rudney, 1943). We have not been able to find an inhibitor of this type, possibly owing to the fact that the group of enzymes designated as ali-esterases includes a wide variety of different types of esterase. However, the results obtained do indicate some of the factors which govern the selectivity towards a given esterase.

In the first place, it might be noted that the carbamate-resistant ali-esterase of *Myco. phlei* is readily inhibited by most of the organophosphorus compounds listed in Table 1. The first two compounds, TEPP and DFP, which have been extensively studied in connexion with their inhibitory activity against the cholinesterases, are potent inhibitors of the ali-esterase of liver (Webb, 1948; Wilson, Levine & Freiberger, 1952; Myers & Mendel, 1953). However, these compounds have considerably less activity against the ali-esterases of brain and of the saprophytic mycobacteria with the exception of *Myco. phlei* (Table 1). The esterase of *Myco. phlei* would, in this respect, appear to be more closely related to the ali-esterase (B-type esterase) of liver than the ali-esterases of the other mycobacteria; a similar conclusion was reached from a study of the substrate specificity of these esterases (Myers *et al.* 1957). The reason for the rapid inhibition of the ali-esterases of liver and *Myco. phlei* by organophosphorus compounds is not known; however, it is to be expected that most, if not all, of the organophosphorus compounds which may be used as selective inhibitors of other ali-esterases will also inhibit the esterases of *Myco. phlei* and of liver.

The selection of inhibitors for study by previous investigators has been based largely on the toxicity of the compound and its inhibitory activity against the cholinesterases. To obtain a selective inhibitor of an ali-esterase it was necessary to search for reactive compounds which might have a low inhibitory activity towards the true cholinesterase. For this purpose we were interested in determining the extent to which the same factors govern the activity of the inhibitor against the ali-esterase and the cholinesterase.

The inhibition of the true cholinesterase by organophosphorus compounds has been formulated as a reaction of the type

$$\underset{\text{(inhibitor)}}{R_1R_2\text{—}\overset{\overset{\uparrow}{\mathrm{O}}}{\mathrm{P}}\text{—}X} + \underset{\text{(enzyme)}}{E\mathrm{H}} \rightarrow \underset{\text{(inhibited enzyme)}}{R_1R_2\text{—}\overset{\overset{\uparrow}{\mathrm{O}}}{\mathrm{P}}\text{—}E} + \underset{\text{(acid product)}}{\mathrm{H}X}$$

which involves a primary attack by a group in the active centre of the enzyme on the electrophilic phosphorus atom of the inhibitor (cf. Burgen & Hobbiger, 1951; Wilson, 1951; Aldridge, 1953*b*). An analogous reaction mechanism has been suggested for the inhibition of esterases by sulphonyl and carbamoyl fluorides (Myers & Kemp, 1954; Myers, 1956*a*), and the reaction with the substrate also follows the same general pattern (Nachmansohn & Wilson, 1951).

Aldridge & Davison (1952*a*, *b*) and others have concluded that the rate at which the true cholinesterase is inhibited by the members of a homologous series of organophosphorus compounds parallels the reactivity of the P–*X* bond. In a series of diethyl phenyl phosphate derivatives, the effect of various substituents on the phenyl ring in increasing the inhibitory activity was found to be proportional to their effect in increasing the rate of spontaneous hydrolysis of the P–*X* bond in aqueous solution (Aldridge & Davison, 1952*a*). This relationship has been confirmed with other substituents (Myers, 1954) and seems to hold for all of the ali-esterases tested in the present investigation as well as for the true cholinesterase. The inhibitory activity of the series of *N*-methyl diphenylphosphoramidates with different substituents in the *para* position on the phenyl rings increases in the order expected, i.e. $CH_3 < H < Cl < CO.OCH_3 \leqslant NO_2$ (Table 1, compounds 32, 31, 30, 29 and 20 respectively).

Other *X* groups did not appear to offer any advantage over the *p*-nitrophenyl group in our search for selective inhibitors of the ali-esterases. Substitution of fluoride for the *p*-nitrophenyl group (compounds 2 and 10 respectively) decreased the inhibitory activity slightly without appreciably affecting the selectivity towards the mycobacterial esterases (Table 1). The single chloro derivative tested (compound 5) was considerably less active than the corresponding fluoro derivative (compound 4); most of the other phosphoryl chloride derivatives are too rapidly hydrolysed in aqueous solutions to be of any value as esterase inhibitors. The pyrophosphate derivative (compound 1) was considerably less active against most of the ali-esterases and

more selective for the true cholinesterase than was the corresponding *p*-nitrophenyl derivative (compound 9). The effect of anionic and cationic substituents in the benzene ring in place of the nitro group has been discussed previously (Myers, 1953, 1954; cf. Wilson *et al.* 1952); these substituents did not appear to offer any advantages in the present investigation. Consequently, most of the remaining organophosphorus derivatives tested contained a *p*-nitrophenyl group.

However, in studying the effect of alterations in the *R* groups, it was frequently more convenient to synthesize compounds containing two *p*-nitrophenyl groups; one of these can be regarded as the *X* group and the other, therefore, as an *R* group in the general formula indicated above. The rate of spontaneous hydrolysis of the P–*X* bond in aqueous solution increases markedly when a second and third *p*-nitrophenyl group is introduced into the phosphate ester (cf. Ketelaar *et al.* 1952). However, the apparent inhibitory activity of the compound does not increase proportionately (Table 1). In fact, the inhibitory activity seems to decrease considerably when the third *p*-nitrophenyl group is introduced (compound 11). Aldridge & Davison (1952*b*) encountered a similar anomaly in their series of compounds when the lability of the inhibitor was altered by other means than by an alteration in the substituent on the benzene ring of the *X* group, e.g. when the *S*-*p*-nitrophenyl group was substituted for the *p*-nitrophenyl group in E 600. One of the factors contributing to these anomalies may be the fact that the compound is rapidly destroyed in the aqueous reaction mixture before it has time to inhibit the esterases. Under these conditions, the apparent pI_{50} value which is measured would be too low; however, this factor should not affect the selectivity of the inhibitor towards the various esterases.

The character of the *R* groups has a marked influence on the selectivity of the inhibitor. Aldridge (1953*a*) has presented evidence in support of the theory that the size and shape of the *R* groups have a similar influence on the selectivity of the inhibitor towards the cholinesterases and on the rate of hydrolysis of choline esters by the same enzyme. However, it might be noted that the rate of hydrolysis of choline esters by the true cholinesterase is probably limited by the rate of hydrolysis of the acyl residue from the active centre of the enzyme (Wilson, 1952; cf. Myers, 1954; Suzuoki-Ziro & Suzuoki-Tuneko, 1954); this reaction is analogous to the reversal of inhibition by hydrolysis of the phosphorylated derivative of the enzyme (cf. Aldridge, 1953*b*) and is directly opposed to the process of inhibition. Moreover, the data given by Aldridge (1953*a*) show much greater alterations in the selectivity when the type of *R* group is altered than when the size is altered. The same factor was emphasized by the results obtained in the present investigation (Table 1). For example, a comparison of the results given for compounds 2 and 3, which are approximately isosteric, shows that the selectivity towards all of the ali-esterases is increased markedly when the *iso*propoxy groups of DFP are replaced by *iso*propylamido groups. The inhibitory activity towards the true cholinesterase decreases markedly, whereas the inhibitory activity towards the ali-esterases only shows a slight decrease when the oxygen atoms adjacent to the phosphorus are replaced by less electronegative nitrogen atoms (Table 1). Although steric factors must have an important influence on the selectivity of an inhibitor, it appears that alterations in the electronic pattern may have a greater influence. It will therefore be difficult to interpret the influence of steric factors on the selectivity of these inhibitors.

However, if we compare the results obtained with various organophosphorus compounds which are approximately isosteric, it becomes evident that alterations in the electronic pattern have less influence on the inhibitory activity towards the ali-esterases than on the inhibitory activity towards the true cholinesterase. This was noted with compounds 2 and 3 above and is also apparent from the results presented in Table 1 for compounds 18–22, compounds 23–25 and compounds 26 and 27. The results obtained with compounds 7 and 8 and compounds 11–17, in which more than one *R* group is varied, also show a tendency in the same direction but are less consistent. In most cases, the members of each series of isosteric compounds all exhibit approximately the same inhibitory activity towards a given ali-esterase, whereas the inhibitory activity towards the true cholinesterase varies over a considerable range.

The selectivity of these inhibitors towards the true cholinesterase usually decreases in the following order: (R_2=) *R*–O–, *R*–S–, *R*–NH– = *R*–CH_2– (Table 1). The electronegative character of these groups, i.e. their ability to attract electrons from the phosphorus atom to which they are attached, decreases in the same order (cf. Johnson, 1942). The influence of this inductive effect of the *R* group is even more striking in compounds 26 and 27 in which R_2 is a *cyclo*hexane and a benzene ring respectively. Both compounds inhibit the ali-esterases to about the same extent, but compound 27 is at least 2000 times more active than compound 26 against the true cholinesterase (Table 1). In this connexion it should be noted that the effect of the *R* group on the selectivity of the inhibitor towards the true cholinesterase probably reflects the polarizability of the P–*R* bond during the reaction with the cholinesterase as well as the permanent polarization which is induced in this bond.

The results discussed above agree well with the theory of cholinesterase inhibition outlined by Wilson (1951), but it is necessary to assume that the inhibition of the ali-esterases follows a different reaction mechanism. Undoubtedly the inhibitory reaction with the ali-esterases also involves the formation of a phosphorylated derivative of the active centre of the enzyme since the inhibitory activity of the organophosphorus compounds is highly dependent on the lability of the P–*X* bond as noted above; moreover, the inhibition is practically irreversible in certain cases (cf. Mendel & Myers, 1953) and phosphorylserine derivatives have been isolated from a preparation of inhibited ali-esterase as well as from inhibited cholinesterase (Cohen, Oosterbaan & Warringa, 1955). However, it seems unlikely that the inhibitory reaction with the ali-esterases involves a primary attack by a neutrophilic group in the enzyme on the phosphorus atom of the inhibitor, as postulated for the true cholinesterase.

It might be noted that the results reported by Aldridge (1953*a*) for the selectivity of inhibitors towards true cholinesterase as compared with pseudocholinesterase tend to follow the same general pattern as the results reported in Table 1. This would suggest that the mechanism of the reaction between the organophosphorus compounds and pseudocholinesterase may be similar to that with the ali-esterases. Ormerod (1953) has suggested that the reaction between the substrate and pseudocholinesterase involves a primary attack on the oxygen atom of the carbonyl group. A similar type of reaction between the enzyme and the oxygen atom of the phosphoryl group of the organophosphorus compounds would provide a possible explanation for the above results.

Consideration of the results obtained with compounds 39–41 and 45–49 indicates that the true cholinesterase must be inhibited in a different way by these carbamate esters. The experimental evidence obtained previously showed that the inhibition of the pseudocholinesterase by a prostigmine analogue (Myers, 1952) and of both the cholinesterase and ali-esterase by two carbamoyl fluorides (Myers, 1956*a*) is analogous to the inhibition by the organophosphorus compounds, i.e. the inhibitory reaction involves the formation of a dialkylcarbamoyl derivative of the active centre of the enzyme. However, Kobezen *et al.* (1954) concluded that the inhibition of true cholinesterase by the carbamate esters shown in Table 1 is due to the combination of the intact ester with the enzyme. Our results confirm this conclusion. The inhibitory activity of the *N*-methylcarbamoyl esters listed as compounds 44–48 towards the true cholinesterase decreases in the order *p*-cresyl, phenyl, *p*-chlorophenyl, *p*-nitrophenyl (Table 1). This order is the reverse of that expected if the inhibitory reaction involved a transesterification of the *N*-methylcarbamoyl group from the phenol to the active centre and is the reverse of that observed with analogous organophosphorus compounds. Moreover, the inhibitory activity of all of these compounds towards the true cholinesterase is relatively low; this would agree with the supposition that the inhibition is due to an adsorption of the intact molecule on the enzyme.

Surprisingly, these carbamate esters were found to be much more active against certain of the ali-esterases (Table 1). The explanation of this finding is still uncertain, and further investigation will be required to ascertain the mechanism of this inhibition. It might be noted that these compounds are almost isosteric with the phenyl esters which are rapidly hydrolysed by the ali-esterases (cf. Myers, 1956*b*; Myers *et al.* 1957). However, the carbamate derivatives are relatively stable compounds (Kobezen *et al.* 1954); our own results indicate that the rate of spontaneous hydrolysis of *p*-nitrophenyl *NN*-dimethylcarbamate (compound 49) in aqueous solution at pH 8·6 is approximately 15 000 times slower than the rate of hydrolysis of *p*-nitrophenyl acetate under the same conditions (Myers, 1954). It would be expected therefore that the carbamate esters would be also hydrolysed extremely slowly by the esterases.

The behaviour of the sulphonyl derivatives seems to be analogous to that of the corresponding carbamoyl derivatives. The sulphonyl fluorides, like the carbamoyl fluorides, exhibit a moderate inhibitory activity towards the cholinesterase and ali-esterases (Table 1) (cf. Myers & Kemp, 1954). However, the *p*-nitrophenyl ester of methanesulphonic acid is practically inactive against the esterases of rat brain (Myers, 1954); this behaviour resembles that of the *p*-nitrophenyl carbamate derivatives and contrasts with that of the *p*-nitrophenyl phosphate derivatives. The reason for this difference is not known but must be attributed to differences in the structure of the inhibitor and the mechanism of the inhibition rather than differences in the reactivity of the inhibitor. The ratio of the rates of spontaneous hydrolysis in aqueous solution at pH 8·6 is approximately 40:20:1 for the *p*-nitrophenyl esters of diethyl phosphoric acid (compound 9), methanesulphonic acid and *NN*-dimethylcarbamic acid (compound 49) respectively (Myers, 1954). If one could extrapolate the data of Aldridge & Davison (1952*a*, *b*) from the phosphate esters to include the two last compounds, the ratio of the I_{50} values should show the same relation as the ratio of the rates of spontaneous hydrolysis; actually the ratio of the I_{50} values measured with the true cholinesterase of rat brain is approximately 10^6:1:100 for the three compounds mentioned above.

Although many of the results given in Table 1 still retain an empirical character, a number of the compounds listed appear to be valuable as selective inhibitors of the ali-esterases. Compounds 13, 14 and 17 inhibit all of the ali-esterases more strongly than the true cholinesterase, but the activity of compounds 13 and 14 is not very high. Compounds 19–21 are all selective inhibitors of the particular ali-esterases studied in brain and *Myco. phlei* but do not exhibit a similar selectivity towards the ali-esterases of *Myco.* D and E. Compound 26 is exceptionally selective in its inhibitory activity towards the ali-esterases of the mycobacteria; however, the large branched alkyl group which is attached to the phosphorus atom does not appear to be favourable for inhibition of the brain esterase. If the inhibition of the ali-esterases by organophosphorus compounds does not depend on the inductive effects of the *R* groups as was suggested above, then the effect of the *R* groups on the selectivity of the inhibitor towards one ali-esterase as compared with another (rather than with the true cholinesterase) should depend mainly on steric factors. It would appear that the inhibitory activity of the organophosphorus compounds towards the ali-esterases of *Myco.* D and E is not favoured by a small *R* group as in compounds 19–25; this result does not parallel the substrate specificity patterns observed previously (Myers *et al.* 1957).

In addition to the organophosphorus compounds mentioned above, Table 1 includes a series of carbamate esters which could be used as selective inhibitors of the ali-esterases of *Myco.* D and E. The most promising member of the limited series of compounds tested in the present investigation would appear to be phenyl *N*-propylcarbamate (compound 42). These carbamate esters differ from the organophosphorus compounds in that they do not exhibit any appreciable inhibitory activity towards the type of ali-esterase found in rat brain and only exhibit a moderate activity against the liver ali-esterase (cf. Myers *et al.* 1957).

There is always a possibility that complications would be encountered in the application of these selective inhibitors in experiments *in vivo*. For example, the compound might be altered in the animal body into a derivative which exhibits a different inhibitory activity and possibly a different selectivity. This type of alteration has been observed repeatedly in previous investigations. However, preliminary experiments did not reveal any significant degree of inhibition of the cholinesterases after injection of the compounds 26 and 42. Moreover, we have been successful in producing a selective inhibition of the ali-esterase of rat brain *in vivo* after injection of compounds 19 (Myers *et al.* 1952), 13 (Myers, 1954), 17, 21 and 26 (unpublished experiments).

SUMMARY

1. A series of organophosphorus compounds and carbamate esters has been investigated in connexion with their inhibitory activity towards the esterases of rat brain and of saprophytic mycobacteria *in vitro*.

2. The factors affecting the inhibitory activity and selectivity of these compounds have been discussed and correlated with the differences in the mechanism of the inhibitory reaction. Although many of the compounds investigated are poor inhibitors of true cholinesterase, they may be highly active against ali-esterases.

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A Study of the Uptake of Silicon and Phosphorus by Wheat Plants, with Radiochemical Methods

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The limit of the classical gravimetric method for the determination of silicon is approximately 100–1000 μg., weighed as silica. With the development of absorptiometric methods, which use either the yellow colour of the silicomolybdate complex or the blue colour obtained on reduction, the lower limit is approximately 10 μg. A recent paper by Miller & Chalmers (1953) describes a sensitive microgravimetric method which is based on the precipitation of the quinoline salt of the heteromolybdate and gives a sensitivity of a few micrograms. The application of these methods in biological and medical investigations is somewhat restricted owing to the frequently encountered presence of phosphates, which form similar complexes with molybdate, and to the presence of other organic and inorganic substances which interfere with the methods. Though attempts have been made to overcome these difficulties, e.g. by bleaching the phosphate complex with citrate, oxalate or tartrate (Sesa & Rogers, 1954*a*), adding excess of phosphate (Krummholz, 1933), or by extracting with organic solvents (Wadelin & Mellon, 1953; Sesa & Rogers, 1954*b*), the methods are not applicable to the exact determination of very small quantities of silicon in the form of silica or silicate.

Holt & Yates (1953) have used radioactive silicon, ^{31}Si, to determine the silicon content of organs in mice and rats by injecting the animals with active silicate solution and following the distribution in the body for a period of about 6 hr.

Previous work on the uptake of silicon by plants (Hull & Morison, 1906; Jennings, 1919; Nemec, 1927; Whittenberger, 1945) deals with the absorption from soils by mature plants and the published figures only give a measure of the total silicon content in the later stages of development. It was hoped to show that silicon is readily absorbed in the very early stage of growth and to determine the rate of uptake over short intervals of time. It was also desirable to study the influence of added phosphate on the silica uptake.

There appears to be no published work in which radioactive silicon has been used to study the rate of silicon uptake by plants. Experiments are reported in this paper which show that it is possible to follow the uptake of silicon by wheat plants for at least 15 hr. by the use of radioactive silicon, even though the half-life of ^{31}Si is very short (157 min.) and the rate of uptake is comparatively small. The accuracy of the method is of the order of 0·5 μg. of silica 6 hr. after taking from the pile.

To facilitate comparison, experiments are described in which radioactive phosphorus is used to determine the rate of absorption of phosphate by similar plants, and to show the influence of added silicate on the phosphate uptake.

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METHODS

Preparation of ^{31}Si-*labelled silicate solution.* Rock crystal containing at least 99·95% of silica was powdered and irradiated in the Harwell pile for 3 days to produce ^{31}Si. Best results were obtained by irradiating the sample in an aluminium container rather than in a fused-silica tube because, when the silica tube was opened, fine particles from it sometimes contaminated the irradiated specimen and introduced traces of longer-lived isotopes of sodium and potassium. The purity of the specimens irradiated in aluminium containers was shown by determining the specific activity over several hours, from which the half-life was found to lie between 149 and 157 min. On account of the short half-life of ^{31}Si it was necessary to perform all operations with the minimum delay.

The specific activity of each supply of irradiated silica depended upon the pile factor used for each irradiation and upon the time interval which elapsed while the material was being transported from Harwell and prepared ready for use in the laboratory. It was therefore necessary to prepare a calibration curve of each consignment.

Irradiated silica (12·5 mg.) was fused with Na_2CO_3, the melt dissolved in water and the volume made up to 1 l. Portions of this solution were withdrawn and made up to a standard volume of 10 ml. in the liquid counter designed by Veall (1948) and their activities determined with an EHT unit, scaler and dekatron-timing unit. In each case the actual time of counting was recorded so that allowance could be made for the loss of activity due to radioactive decay. Allowance was also made for lost counts and background counts in the usual manner.

Table 1 shows the counting rate and background-counting rate obtained with four consignments of irradiated silica.

The figures indicate that about 0·5 μg. of silica may be estimated 6 hr. after removal from the pile. The sensitivity will decrease with time and will be about 0·25 μg. 3·5 hr. and about 1 μg. 8·5 hr. after removing the silica from the pile. The loss in activity due to decay is partially compensated by the increase in the uptake of silica by absorption by the plant.

Germination of the wheat seeds. Wheat seeds (*Triticum*) were germinated in closed Petri dishes on wet filter paper for 3–4 days. The roots of the young plants were then inserted through holes in paper covering a beaker which contained nutrient solution (see Fig. 2). Care was taken to immerse only the roots. The nutrient solution consisted of 1·21 g. of $Ca(NO_3)_2,4H_2O$, 0·275 g. of KCl, 0·275 g. of $MgSO_4,7H_2O$, 0·011 g. of NaCl, in 1 l., and was renewed three or four times. Light and air were provided. After 3 weeks, plants which showed similar development were selected for the experiments.

Assay of the uptake of silica. After taking the plants from the culture beakers they were either (*a*) transferred to a beaker containing 400 ml. of the same nutrient solution equivalent to 12·5 mg. of active silica/l. or (*b*) washed free from nutrient solution and then placed in 400 ml. of an alternative solution containing 0·5 g. of KNO_3, 0·275 g. of KCl, 0·011 g. of NaCl, in 1 l., to which was added the same amount of silica. Both solutions were adjusted to pH 5·5. The alternative solution was used to show that the activity found in the roots was not caused by precipitation of calcium and magnesium silicates, no detectable difference was found in the uptake rate from each solution. Throughout the experiments the illumination was kept constant at 500 lux.

After varying intervals, two or three plants were withdrawn from the solution and carefully washed with water to remove any adsorbed active material. They were then dried in a current of warm air, weighed (air dry), ashed at as low a temperature as possible, and then fused with 500 mg. of Na_2CO_3 in a platinum crucible. The resulting mass was dissolved in dilute HCl, transferred to the liquid counter and diluted to the standard volume. The proportion of silica taken up by the plants was determined by comparing the counting rates with those of standard solutions. Allowance was made for background, and lost counts were taken into consideration in the usual way.

Assay of the uptake of phosphate. A series of experiments was carried out to determine the rate of uptake of phosphate, by a similar method to that utilized in the silicate experiments. A sufficient amount of carrier-free ^{32}P as phosphate (obtained from the Radiochemical Centre, Amersham) was added to the nutrient or the alternative solutions containing 30 mg. of KH_2PO_4/l., to give approximately the same initial specific activity as the silica solutions.

The proportion of phosphate taken up by the plants after varying intervals was determined by the same procedure, but no detectable difference was found in the uptake rate from each solution. The counting rates of the solutions were compared with standard solutions and allowance was again made for background and lost counts.

RESULTS

Uptake of [^{31}Si]*silicate without added phosphate.* The absorption by wheat plants of [^{31}Si]silicate from a solution free from phosphate ions was determined by the radiochemical method described

Table 1. *Counting rate and background-counting rate of four consignments of irradiated silica*

The solution (10 ml.) in the liquid counter contained 12·5 mg. of irradiated SiO_2/l. The pile factor of each irradiation is shown, together with the time of determining the activity. In each case, the sample was withdrawn from the pile at approx. 7.30 a.m.

Date	Pile factor	Time (p.m.)	Background count (counts/min.)	Corrected count (counts/min.)	Calc. counts/min./ μg. of SiO_2	Ratio of counts/min./μg. of SiO_2 to background
7 July	14	1·26	18	6407	51·3	2·85:1
14 July	12	2·17	20	7018	56·1	2·81:1
21 July	12	12·25	26	7310	58·5	2·25:1
11 August	12	12·23	23	6243	50·0	2·17:1

Table 2. *Typical results of uptake of silica by wheat plants from solutions free from phosphates with* ^{31}Si

Plants were withdrawn from the solution and their combined leaf–root systems worked up separately as indicated.

Specimen no.	Time in soln.	No. of plants	Position of cut above seed (cm.)	System	Weight (mg.)	Counts/min. (corrected)	Counts/min./g. of plant	SiO_2/g. of plant (μg.)
C4	4 hr.	2	2·5	Leaves	792·5	1278	1610	31·4
				Roots	465·6	1540	3310	65·0
				Whole plant	1258·1	2818	2240	43·4
C9	11 hr. 50 min.	2	10·0	Leaves	435·4	1480	3400	66·0
				Roots	861·5	7900	11900	229·0
				Whole plant	1297·0	9380	7210	139·8

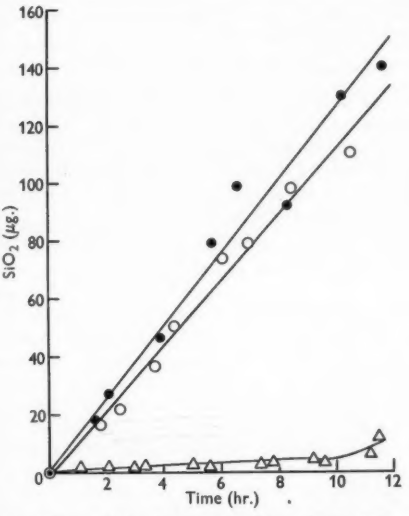

Fig. 1. Silica uptake by young wheat plants from the nutrient solution containing 12·5 mg. of silica/l. Uptake referred to 1 g. of plant, 18 days old: temp., 23°. ●, Solution free from phosphate; ○, solution containing 30 mg. of KH_2PO_4/l.; △, solution containing 75 mg. of suspended quartz/l.

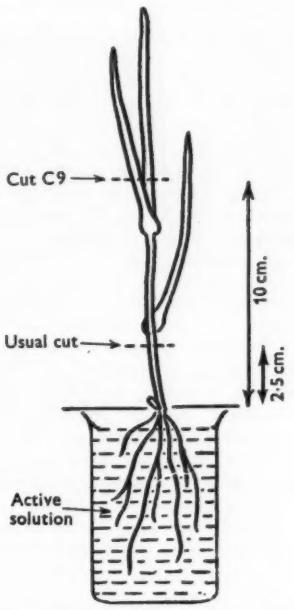

Fig. 2. Illustration of the setting up and cutting position of plant. The roots only are immersed in the radioactive solution.

above. The amount of absorbed silica/g. of plant was calculated after varying intervals (Fig. 1). The scattering of points may be caused by small deviations in the sizes of the roots and the leaves of the plants. The average uptake of silica by the wheat plants under the described conditions is seen to be 12·4 μg./g. of plant/hr.

To confirm that true absorption of the silica had taken place, a number of the plants were cut into two sections and the activities of both the root system and the leaves determined separately. Fig. 2 indicates the mounting and the cutting of the plants. It was found that the average content of silica/unit weight of the leaves was approximately half that of the roots. Some typical results are shown in specimen C4, Table 2.

Specimen C9 (Table 2) illustrates an experiment in which the cut was made 10 cm. above the paper (Fig. 2). It is clear that the amount of silica present in the upper portion is smaller, but nevertheless significant.

Uptake of [^{31}Si]*silicate in the presence of phosphate.* The absorption of [^{31}Si]silicate in the presence of phosphate by wheat plants taken from the same batch, at the same time and under the same

conditions as those used in the previous experiments, is shown in Fig. 1. The average uptake of silica can be seen to be 11·2 μg./g. of plant/hr. This represents a decrease of 10 % due to the presence of an equimolar amount of P_2O_5.

Uptake of silicon from solutions containing suspended quartz. Irradiated powdered quartz (75 mg.) was dispersed in 1 l. of nutrient solution and the plants were introduced as described above, the solution being occasionally stirred. The average size of the quartz particles was 6 μ. The uptake of silica from these solutions is shown in Fig. 1 and, on an enlarged scale, in Fig. 3. It will be seen that the rate of uptake is very small compared with that obtained with the [^{31}Si]silicate and is of the order of 0·3 μg./g. of plant/hr.

Activity was again detected in the leaves and roots, and after several hours the slope of the uptake curve became steeper. Preliminary experiments to be described fully elsewhere indicate that this may be due to an increase in the rate of dissolution of the silica. Alternatively, Holzapfel & Engel (1956) suggest that microcrystalline quartz particles are absorbed by the roots of the seedlings and ultimately migrate through the plant and enhance the original silica content due to silicate.

Uptake of phosphate without added silicate. The absorption of phosphate by wheat plants from solutions free from added silicate was determined by the radiochemical method outlined above and the uptake of phosphate calculated as P_2O_5, after various intervals, is summarized in Fig. 4. An average uptake of 16·5 μg. of P_2O_5/g. of plant/hr. can be calculated. Similar values were obtained from both nutrient and alternative solutions to which had been added similar amounts of phosphate. The activity was again distributed throughout the leaves and the root systems.

Uptake of phosphate in the presence of silicate. The absorption of phosphate in the presence of [^{31}Si]silicate was followed with plants of the same lot and under the same conditions as those used in the experiments reported in the previous section.

The average uptake calculated from this curve (Fig. 4) is 22·2 μg. of P_2O_5/g. of plant/hr., an increase of 25 % over the solutions without silicate.

Statistical analysis of results. The regressions of SiO_2 uptake on time have been fitted by the method of least squares with the following results (the lines are shown in Fig. 1):

	Without phosphate	With phosphate
Equation of regression	$y = 12{\cdot}4x + 0{\cdot}8$	$y = 11{\cdot}2x - 1{\cdot}5$
Slope of regression line	12·39	11·22
Standard error of slope	0·75	0·58

The t test for the difference between the slopes gives the following value:

$$t = 12{\cdot}39 - 11{\cdot}22/\sqrt{(0{\cdot}75^2 + 0{\cdot}58^2)} = 1{\cdot}3$$

with 14 degrees of freedom. This corresponds to a probability of about 0·2 (20 %) that the difference is only caused by statistical scattering. Thus although the difference between the slopes of the two lines in Fig. 1 is not statistically secured, there is a typical trend to form a difference.

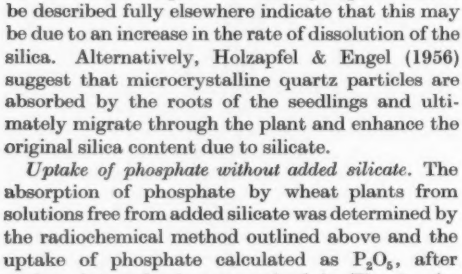

Fig. 3. Silica uptake by young wheat plants from the nutrient solution containing 75 mg. of suspended quartz/l. Uptake referred to 1 g. of plant, 18 days old; temp., 23°. ●, Solution free from phosphate; ○, solution containing 30 mg. of KH_2PO_4/l.

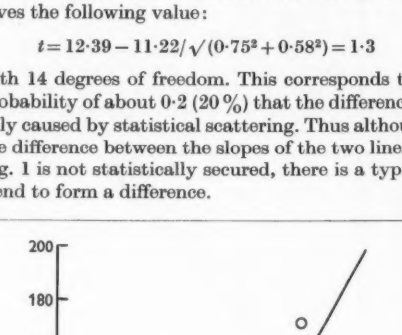

Fig. 4. Uptake of phosphate calculated as P_2O_5 by young wheat plants from the nutrient solution containing 30 mg. of KH_2PO_4/l. Uptake referred to 1 g. of plant, 19 days old; temp., 23°. ●, Solution free from silicate; ○, solution containing 12·5 mg. of SiO_2/l.

Similarly, the regressions of P_2O_5 uptake on time have been fitted and the lines are shown in Fig. 4:

	Without silicate	With silicate
Equation of regression	$y = 16{\cdot}5x + 2{\cdot}4$	$y = 22{\cdot}2x - 7{\cdot}2$
Slope of regression line	16·50	22·24
Standard error of slope	1·52	2·16

The t test gives a value of 2·2 with 9 degrees of freedom, corresponding to a probability of about 0·05 (5%). These figures tend to show that the difference in the slopes of the two lines is statistically significant.

DISCUSSION

The results show that the uptake of silicon by young wheat plants and calculated as silica is of the same order as that of phosphorus calculated as P_2O_5. However, when it is taken into consideration that P_2O_5 contains two atoms of phosphorus, the uptake ratio, i.e. the ratio of the number of silicon atoms to phosphorus atoms, is about one to three.

From the figures given in Table 2 it can be concluded that there has been true absorption of [^{31}Si]silicate. The solution contained only 12·5 μg. of silica/ml., yet after approximately 12 hr. the concentration of silica in specimen C9, for example, had increased sixfold in the top leaves, 20-fold in the roots and an average of tenfold in the whole plant.

The average uptake of silicon and phosphorus/g. of plant under the condition of the experiments and calculated from regression forms are: SiO_2 from solutions free from phosphate, 12·4 μg./hr.; SiO_2 from solutions containing phosphate, 11·2 μg./hr.; P_2O_5 from solutions free from silicate, 16·5 μg./hr.; P_2O_5 from solutions containing silicate, 22·2 μg./hr.

The presence of phosphate appears to have a quenching effect on the uptake of [^{31}Si]silicate though this is not yet statistically secured. This may be explained by assuming that the extra amount of silica which is taken up in the absence of phosphate is used instead of phosphate (cf. Holzapfel, 1951, 1954). On the other hand, the uptake of phosphorus is slightly stimulated in the presence of silicate. These results appear at first to be conflicting, but it will be noticed that the total silicate and phosphate uptakes are approximately constant. Consequently, a decrease in the silicate uptake will cause an increase in the phosphate uptake and vice versa, and the close connexion of their metabolism as observed in other systems becomes evident.

The influence of silicate on the uptake of phosphorus in the early stages of growth and under the conditions given above is, however, not as significant as that reported in an early publication (Hull & Morison, 1906) when the effect of the combined addition of phosphate and silicate on the field crop yield of cereals was investigated.

Further experiments are planned to investigate the influence of other compounds on silicon uptake of different model organisms and to evaluate the role of silicon in plant and animal metabolism. It is hoped that later when the longer-lived radioactive isotope of silicon becomes available long-term experiments may be undertaken.

SUMMARY

1. The described technique involving the use of ^{31}Si is able to determine silica in the form of silicate or SiO_2 with an accuracy of 0·5 μg.

2. It has been shown that even young wheat plants have a significant capacity to absorb silica. After only half an hour (the shortest duration of experiment) considerable amounts were detected in various parts of the plants. The distribution of the absorbed silica over the whole plant was followed.

3. Added phosphate slightly depressed the uptake of silicate, and added silicate enhanced the absorption of phosphate, showing the close relation of their metabolism.

The authors would like to express their appreciation to Mr J. A. Waddams for his co-operation and to Dr L. Holzapfel of the Max Planck Institut, at whose instigation the work was carried out.

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The Amino Acid Sequence in a Hypertensin

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The isolation of a hypertensin peptide, obtained by the action of rabbit renin on ox serum, was described recently (Peart, 1956). The amino acid analysis was consistent with a decapeptide structure involving eight different amino acids, which were leucine (Leu) (1), aspartic acid (Asp) (1), arginine (Arg) (1), phenylalanine (Phe) (1), proline (Pro) (1), tyrosine (Tyr) (1), histidine (His) (2) and valine (Val) (2).

The present communication describes experiments which have led to the elucidation of the sequence of amino acid residues. A number of different techniques were applied simultaneously; they included partial acid hydrolysis, end-group determination and digestion by various enzymes; these can now be regarded as standard procedures in this type of work (see, for example, Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). In addition, the stepwise method of degradation devised by Edman (1950) was employed. We were unable to utilize aminopeptidase (Smith & Spackman, 1955) for stepwise degradation, but, as shown below, this enzyme proved to be useful in other directions. A preliminary account of this work has already been published (Elliott & Peart, 1956).

METHODS

Hypertensin

Prepared as previously described by Peart (1956), this was contaminated with inorganic material in spite of very careful washing of the supporting media used in the columns. These inorganic impurities interfered with the electrophoretic separations carried out on enzymic digests. They were removed as follows. An amount of hypertensin equivalent to 1 mg. of active peptide was dissolved in 0·5% trichloroacetic acid solution (2 ml.; prepared from redistilled trichloroacetic acid) and extracted twice with peroxide-free *n*-butanol (2 ml.). The combined butanol layers were diluted with peroxide-free ether (8 ml.) and the solution was extracted twice with water (4 ml.). The combined aqueous layers were acidified with 6N-HCl (0·05 ml.) and extracted continuously with ether (250 ml.) in the apparatus described by Elliott & Crawhall (1955). The aqueous solution was then evaporated to dryness in a vacuum desiccator. The recovery of hypertensin was quantitative.

Paper chromatography

For most work Whatman no. 3 paper was used. Paper used for isolation of peptides was washed for several days in a chromatography tank with 4N acetic acid and then with water. Phenol–water was prepared by adding 5 vol. of liquid phenol to 2 vol. of water. It was used in a tank containing NH_3 vapour and a small quantity of KCN was dissolved in it immediately before use. The butanol–acetic acid mixture was prepared by shaking together *n*-butanol, water and acetic acid (63:27:10, by vol.) and keeping for several days before using the upper layer. Chromatograms were dried in a current of air at 40°, sprayed with a 0·1% ninhydrin solution in *n*-butanol containing 2% of acetic acid, moistened by steaming and heated at 90°. Chromatograms of peptides from which spots were to be cut for subsequent end-group determination or hydrolysis were sprayed with 0·025% ninhydrin solution, moistened by steaming and heated at 40° until colour development had reached a maximum. The isatin reaction described by Acher, Fromageot & Jutisz (1950) was used for the detection of proline.

Chromatography of dinitrophenyl amino acids (DNP amino acids) was carried out on Whatman no. 1 paper with the *tert.*-pentanol system of Blackburn & Lowther (1951). This one-dimensional system was adequate for the separation of the DNP derivatives of the amino acids in hypertensin.

Electrophoresis

This was carried out on strips of Whatman no. 3 MM paper, 45 cm. × 12·5 cm., in an uncooled apparatus similar to that described by Durrum (1950). The paper was washed with 4N acetic acid before use. Acetic acid (2N) was used throughout as the buffer; the potential gradient was 15 v/cm. and the current was approximately 10 mA. Substances were applied to the centre of the dry paper in the form of a narrow band varying from 0·5 to 2·5 cm. in length, according to the quantity of sample (0·25–1·0 mg.). At the end of the run the paper was dried in a current of warm air. A strip of approximately 2 mm. width was cut from the centre and along the length of the paper and stained by one of several methods. These included the usual ninhydrin method, the Pauly reagent of Ames & Mitchell (1952) and the chlorine method of Rydon & Smith (1952) as modified by Reindel & Hoppe (1954). The two unstained portions of the paper were marked accordingly and the parts containing the desired material cut and eluted by the technique described by Sanger & Thompson (1953*a*); 2–3 ml. of eluate was generally collected from these strips.

Determination of end groups by the fluoro-2:4-dinitrobenzene method

In most cases the method of Sanger & Thompson (1953*a*) was used, except that the amounts of trimethylamine and ethanol were increased to 0·2 and 0·4 ml. respectively/0·01 ml. of fluoro-2:4-dinitrobenzene (FDNB). These quantities of reagent were considered satisfactory for 0·5 mg. or less of peptide. With hypertensin itself, however, this technique had previously given poor yields of DNP-aspartic acid (Peart, 1956), and it was necessary to use $NaHCO_3$ as a buffer (Sanger, 1945) to improve the yield. The DNP peptides were hydrolysed with 6N-HCl at 105° for 12 hr. The usual ether extractions of the hydrolysate were replaced by those of ethyl acetate; this resulted in the extraction of bis-DNP-histidine which remains in the acid layer (Sanger, 1945) unless continuous ether extraction is used (Mills, 1952). The DNP amino acids were identified by paper chromatography. In quantitative experiments the spots of DNP amino acids were eluted with 1% $NaHCO_3$ and estimated at 350 mμ. in the Hilger Uvispek spectrophotometer. To correct for losses a known quantity of the DNP amino acid was subjected to chromatography and eluted under the same conditions.

Enzymic hydrolyses

Carboxypeptidase was an aqueous suspension obtained from Armour Laboratories. Trypsin and chymotrypsin were recrystallized samples kindly supplied by Mr J. Lightbown of this Institute. Aminopeptidase was kindly prepared by Dr R. R. Porter, by the method of Smith & Spackman (1955). Final purification was by zone electrophoresis on a column (40 cm. × 2·6 cm.) with treated cotton wool as supporting medium (Flodin & Porath, 1954; Porath, 1954; Campbell & Stone, 1956). Buffer solutions for carboxypeptidase, chymotrypsin and trypsin digestions were prepared by mixing M solutions of ammonium acetate and NH_4HCO_3 to give the required pH as shown by the glass electrode, and diluting to 0·05M. Carboxypeptidase digestions were carried out at pH 8 at room temperature or 37°. The enzyme:substrate molar ratio was generally about 1:80, but in some cases a ratio of 1:40 was used. Immediately before use any proteolytic impurities which may have been present were poisoned by the addition of di*iso*propyl phosphorofluoridate (DFP) (cf. Sanger & Thompson, 1953*b*). For example, 0·028 ml. of a suspension of carboxypeptidase (13 mg./ml.) and 0·5 ml. of 0·002M DFP were added to 3·5 ml. of 0·05M buffer at pH 8. The mixture was incubated at 37° for 30 min., cooled and divided into two equal portions. One portion was used as a control and was treated identically with the second portion, to which was added 0·6 mg. of hypertensin. The mixture was incubated at 37° and 0·5 ml. samples were removed at intervals. To each sample was added 0·05 ml. of acetic acid and the solution evaporated to dryness in a vacuum desiccator over KOH and H_2SO_4. The residue was dissolved in water and applied directly to Whatman no. 3 paper. Chromatograms were run in butanol–acetic acid, a mixture of all the amino acids present in hypertensin being used as markers.

Chymotrypsin digestion was carried out as follows: hypertensin (0·25 mg.) was dissolved in buffer at pH 7·5 (1·5 ml.) and to the solution was added a solution of chymotrypsin (0·016 ml. of a solution containing 4 mg./ml.) in the same buffer. The mixture was incubated for 16–20 hr. at 37°, acidified with 0·1 ml. of acetic acid and evaporated as in the carboxypeptidase digestion. The residue was redissolved in water and evaporated a second time. The products were separated by ionophoresis on 3 MM paper. Quantities of hypertensin up to 1 mg. were handled in a similar way.

Trypsin digestion was carried out in a similar fashion to the chymotrypsin digestion at pH 7·5 and 37° for 18 hr. The products were separated by paper chromatography with butanol–acetic acid.

The amount of aminopeptidase required for digestion was discovered by a preliminary trial. For example, 0·01 ml. of a solution of aminopeptidase was activated by incubation for 40 min. at 37° with 0·1 ml. of 0·1M ammonium acetate–NH_3 soln. buffer at pH 8, made 2·5 mM with respect to $MnCl_2$. This was then added to 0·5 ml. of the same buffer system at pH 8·6, made 2 mM with respect to $MnCl_2$ and containing 0·5 mg. of hypertensin. Such a mixture was incubated at either room temperature or 37° and samples were withdrawn at intervals. A control solution omitted only the hypertensin. The samples were acidified with a drop of acetic acid and dried in a desiccator over NaOH and H_2SO_4. The products of digestion were separated by paper chromatography in butanol–acetic acid.

Partial acid hydrolyses

The peptide was dissolved in HCl (36%, w/w) and incubated at 37° for 3–4 days. The product was evaporated to dryness in a vacuum desiccator and submitted to paper chromatography or electrophoresis.

Edman stepwise-degradation method

For the reaction with phenyl *iso*thiocyanate and subsequent cyclization to give a thiohydantoin, the technique worked out by Fraenkel-Conrat, Harris & Levy (1955) was adapted as follows: 1·2 mg. of hypertensin was dissolved in 50% aqueous dioxan (6 ml.) to which was added freshly distilled phenyl *iso*thiocyanate (0·1 ml.). The mixture was heated to 40° in a thermostat bath, stirred with a stream of N_2 and 0·01N-NaOH added from an Agla microburette to maintain a pH of 9 with the glass electrode. The rate of addition of alkali was plotted graphically and the experiment was stopped when a constant 'end-slope' was reached (see Fig. 1). The solution was evaporated to small bulk under reduced pressure at about 35° to remove most of the dioxan and extracted seven times with redistilled benzene. The aqueous layer was evaporated to dryness in a vacuum desiccator.

The residue was dissolved in 3N-HCl (10 ml.) (prepared from redistilled constant-boiling HCl) and the optical density of the solution measured as rapidly as possible over the range 230–270 mμ. in the Hilger Uvispek instrument at intervals of 5 mμ. The cyclization was considered to be complete when no further change in optical density occurred at 265 mμ. The solution was then extracted five times with ethyl acetate (Spectrosol brand from Hopkin and Williams Ltd., London). The ethyl acetate extracts were combined, evaporated under reduced pressure and the residue was hydrolysed by heating with 0·1 ml. of HI (redistilled; *d* 1·7) at 140° for 2 hr. in a sealed tube. The hydrolysate was evaporated to dryness in a desiccator over KOH and the amino acid identified by chromatography in butanol–acetic acid. The acid solution remaining after ethyl acetate extraction was evaporated to dryness over KOH. The above cycle of operations was then repeated on the residue. After

the third series of reactions, the volume of 3N-HCl used for cyclization was reduced to 5 ml. and the acid solution remaining after ethyl acetate extraction of the phenylthiohydantoin was evaporated to dryness. The residue was redissolved in a small volume of dilute $NaHCO_3$ and submitted to a second extraction with ethyl acetate. This ensured that the phenylthiohydantoin of histidine, which would otherwise remain in the acid solution (Fraenkel-Conrat *et al.* 1955), would be extracted. The ethyl acetate extracts were combined and treated exactly as those from the acid solution. The aqueous solution was made weakly acid with HCl (pH 4) and evaporated to dryness. Reaction with phenyl *iso*thiocyanate could then be carried out on the residue.

Stepwise degradation of the peptide HC2 was performed in a similar fashion, the double-extraction procedure being performed at each stage.

RESULTS

N-Terminal residue of hypertensin

Although a number of attempts were made it was not possible to obtain more than 50% of the theoretical yield of DNP-aspartic acid. Other workers have obtained low yields of DNP-aspartic acid from aspartyl peptides (Schroeder & Le Gette, 1953). It seemed probable, therefore, that an *N*-terminal residue of aspartic acid or asparagine was present in hypertensin.

Hydrolysis of hypertensin by aminopeptidase

Digestion with aminopeptidase for 14 hr. at 37° released all the amino acids present in hypertensin and there was no evidence of peptides remaining undigested. At room temperature, when samples were taken at 0·5, 2 and 6 hr. respectively, all the amino acids appeared simultaneously and seemed to increase in amount at the same rate. The quantities were approximately gauged by visual comparison with serial dilutions of an equimolar solution of the amino acids present in hypertensin, run in parallel on the chromatogram. Although small differences in the rate of release of the amino acids would not be detected, the method was sufficiently sensitive to show that there were not big differences in the relative rates. It was decided that with these methods aminopeptidase was not suitable for determination of the amino acid sequence in hypertensin. However, it was useful in showing that aspartic acid and not asparagine was present in hypertensin since the acid was always liberated by the action of this enzyme. This is consonant with the finding that the quantity of alkali consumed in the reaction of hypertensin with phenyl *iso*thiocyanate was in agreement with the presence of two free carboxyl groups (see Edman procedure below).

Edman degradation of hypertensin

Fig. 1 illustrates the uptake of alkali by 1·2 mg. of hypertensin in the presence of phenyl *iso*thiocyanate. Curves obtained at subsequent stages of the degradation were similar in shape, although the total alkali uptake was variable, often being greater than in the first step. In Fig. 2 are shown the initial

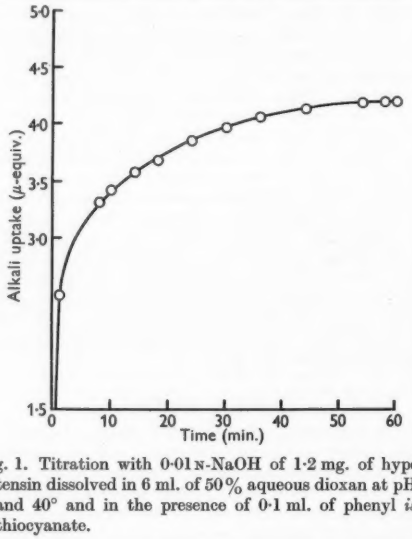

Fig. 1. Titration with 0·01N-NaOH of 1·2 mg. of hypertensin dissolved in 6 ml. of 50% aqueous dioxan at pH 9 and 40° and in the presence of 0·1 ml. of phenyl *iso*thiocyanate.

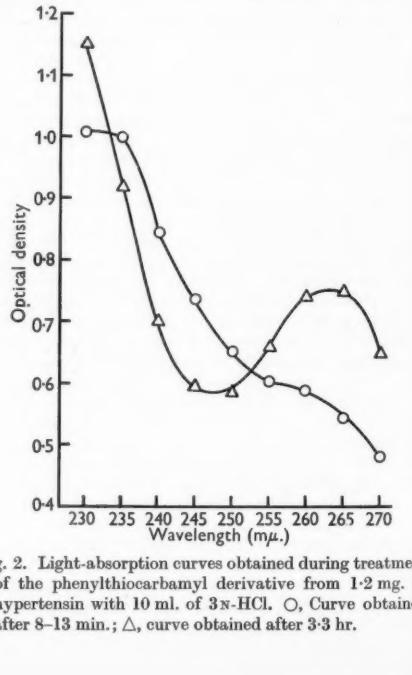

Fig. 2. Light-absorption curves obtained during treatment of the phenylthiocarbamyl derivative from 1·2 mg. of hypertensin with 10 ml. of 3N-HCl. ○, Curve obtained after 8–13 min.; △, curve obtained after 3·3 hr.

Table 1. *Amino acids liberated from hypertensin during various periods of digestion by carboxypeptidase*

Expt. no.	Time (hr.)	Temp.	Relative amounts of amino acids liberated
1	0·25	22°	Leu X
	1		Leu X ? His (trace)
2	2	37	Leu XX Phe X ? His X
	4		Leu XX Phe X ? His X
	8		Leu XXX Phe X ? His X
3	14	37	Leu XXX Phe XX ? His X
	25		Leu XXX Phe XX ? His XX

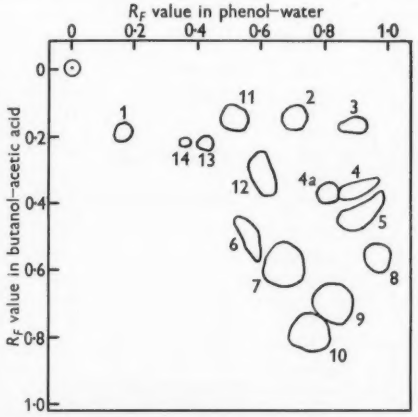

Fig. 3. Chromatogram obtained after partial hydrolysis of 1·1 mg. of hypertensin with HCl (36%, w/w) at 37° for 3–4 days.

and the final light-absorption curves obtained during the first cyclization step. In the subsequent cyclization steps, this characteristic change of shape of the absorption curve did not appear to take place; the only effect observed was a small change in the absorption at 265 mμ. The solution was extracted when no further change occurred. In the first step aspartic acid was obtained, whereas in the second step no recognizable amino acid was detected; the third and fourth steps yielded valine and tyrosine respectively. A total of seven degradations was carried out, but in the fifth, sixth and seventh steps it was not possible to identify any amino acid with certainty owing to the weakness of the spots.

It seemed probable therefore that the *N*-terminal sequence in hypertensin was Asp.Arg.Val.Tyr.... Arginine was placed with less confidence than the other amino acids owing to the fact that its phenylthiohydantoin cannot be extracted from aqueous solution by solvents and its presence is assumed when a thiohydantoin is not found in the ethyl acetate extract after cyclization (Fraenkel-Conrat *et al.* 1955).

C-Terminal sequence in hypertensin

Carboxypeptidase. The results of carboxypeptidase digestion are shown in Table 1. In this and subsequent tables X signifies a readily identifiable spot on a chromatogram, XX has twice the intensity of X, XXX three times the intensity of X, and so on. It was clear that the *C*-terminal amino acid was leucine. The next amino acid in the chain appeared to be histidine, followed by phenylalanine. Histidine was not identified with certainty; in view of its low R_F value in butanol–acetic acid the spot regarded as histidine might have been a peptide consisting of the hypertensin molecule minus one or two *C*-terminal residues.

Partial hydrolysis of hypertensin with acid. Fig. 3 shows the chromatogram obtained from a 3 days' hydrolysate of 1·1 mg. of hypertensin. The results obtained on examination of the spots by total hydrolysis and the FDNB technique are given in Table 2. Spots 11–14 gave on hydrolysis very weak spots of amino acids not present in hypertensin and were probably derived from impurities. The main result was that one dipeptide was isolated in about 60% of the theoretical yield as determined by visual examination of the spots produced by a total hydrolysate. Although this peptide was contaminated with small amounts of other peptides or amino acids (Table 2, spot no. 5) there seemed no doubt that its structure was His.Leu. It then follows that the *C*-terminal sequence in hypertensin is ...Phe.His.Leu. The fact that no other amino acids were liberated from hypertensin after digestion for 25 hr. with carboxypeptidase indicated that proline was connected to phenylalanine (see Smith, 1953), but further proof of this was needed.

Hydrolysis of hypertensin by trypsin

The FDNB technique revealed the presence of two *N*-terminal residues in the trypsin digest of 0·25 mg. of hypertensin. These were aspartic acid and valine. Paper chromatography of a digest of 0·25 mg. of hypertensin run in butanol–acetic acid revealed the presence of a substance of lower R_F value (0·08) than histidine. This was eluted and hydrolysed. Aspartic acid and arginine were the only two amino acids present. The other product of digestion proved

Table 2. *Amino acids and peptides obtained by partial acid hydrolysis of hypertensin*

Spot no.	Relative strengths of amino acids in total hydrolysate			*N*-Terminal amino acid (FDNB method)	Relative strengths of amino acids remaining after removal of *N*-terminal amino acid	Structure
1		Asp XX		Asp	—	Aspartic acid
2		His XX		His	—	Histidine
3	Arg XX	Val X		Arg	Val X	Arg.Val
4	His XX Tyr X	Val XX Phe X	Pro X Leu X	Not found	—	—
4a	Pro XXX	His X	Val X	Not found	—	Peptide + proline
5	His XXXX Pro X	Leu XXXX Val X		His	Leu XXXX	His.Leu
6		Tyr X		Tyr	—	Tyrosine
7		Val XXXX		Val	—	Valine
8	His XX Val XX	Pro XX Phe XX	Tyr XX Leu XX	Not found	—	—
9	Phe X Val (trace)	Leu (trace)		Not found	—	? Phenylalanine
10	Pro XX Leu XXX	Phe XX	Val X	Leu	—	Mixture of peptides + leucine

impossible to identify satisfactorily by paper chromatography. These results, however, indicate the sequence Asp.Arg.Val.... and confirm the *N*-terminal sequence. The sequence Arg.Val had been demonstrated by partial hydrolysis with acid (spot no. 3, Table 2).

Hydrolysis of hypertensin by chymotrypsin

Application of the FDNB technique to a chymotrypsin digest from 0·2 mg. of hypertensin revealed the presence of three *N*-terminal residues. These were aspartic acid, valine and histidine. The peptides in a similar digest could not be separated by paper chromatography. Fig. 4 illustrates the result obtained on paper electrophoresis of a digest of 0·25 mg. of hypertensin. The paper was cut into three sections and stained by different techniques. The experiment was then repeated on the same scale and the bands HC1, HC2, HC3 were eluted and hydrolysed completely. The results are shown in Table 3. In a separate experiment the *N*-terminal residues of HC3 and HC2 were determined by the FDNB method.

*Peptide HC*1. This contained aspartic acid, arginine, valine and tyrosine, and as the *N*-terminal sequence Asp.Arg.Val.Tyr had been previously established for hypertensin, it was clearly the *N*-terminal tetrapeptide. Further confirmation was obtained from the action of carboxypeptidase under various conditions, which revealed the *C*-terminal sequence Val.Tyr (Table 4).

*Peptide HC*2. This contained valine, histidine, proline and phenylalanine, and the *N*-terminal amino acid was shown to be valine by application of the FDNB technique to one-quarter of a batch of

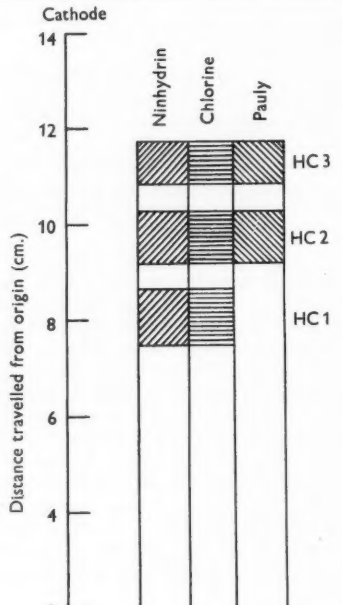

Fig. 4. Result of electrophoresis, for 1·5 hr. in 2N acetic acid at 15 v/cm., of a chymotrypsin digest of hypertensin. Bands stained by various techniques. Scale, ×4 in horizontal direction.

Table 3. *Peptides isolated by electrophoresis of a chymotryptic digest of hypertensin*

Band no.	Relative strengths of amino acids in the hydrolysate	*N*-Terminal residue	Structure
HC1	Asp XX Arg XX Tyr X Val XX	Not determined	Asp.Arg.Val.Tyr
HC2	His XX Pro XX Val XX Phe XX	Val	See Tables 4 & 5
HC3	His XX Leu XX	His	His.Leu

Table 4. *Amino acids liberated from peptides HC1 and HC2 during various periods of digestion by carboxypeptidase*

Expt. no.	Peptide	Time (min.)	Temp.	Enzyme:substrate (molar ratio)	Relative amounts of amino acids liberated
1	HC1	2	20°	1:160	Tyr X
		5	20		Tyr XX
2		15	20	1:160	Tyr XXX Val X
		60	20		Tyr XXX Val XX
3		1080	39	1:40	Tyr XXXX Val XXXX
4	HC2	1080	39	1:40	Phe XXXX

HC2 isolated from 1 mg. of hypertensin. The hydrolysate remaining after removal of the DNP amino acid contained proline, phenylalanine and (iminazole)-DNP-histidine.

Edman degradation carried out on HC2 isolated from 1 mg. of hypertensin indicated the *N*-terminal sequence Val.His.Pro and the action of carboxypeptidase for 18 hr. at an enzyme:substrate ratio of 1:40 showed only phenylalanine (Table 4), which strongly suggested that the adjacent residue was proline (see Smith, 1953).

Partial acid hydrolysis of HC2 derived from 0·75 mg. of hypertensin was carried out for 3 days. Fig. 5 shows the results obtained on electrophoresis of one-half of the product. The remaining material was separated as before, and, after staining a marker strip by the chlorine method, the peptides HC2A1, HC2A2 and HC2A3 were eluted. Table 5 gives the results of hydrolysis of these peptides and of an end-group determination on HC2A3. For the latter purpose, another partial hydrolysis of HC2 was carried out.

Peptide HC2A1 gave only proline and phenylalanine on hydrolysis and was, therefore, Pro.Phe; peptide HC2A2 was clearly unchanged tetrapeptide, and HC2A3 must have been Val.His. The latter sequence was confirmed by the FDNB method. The complete sequence of HC2 was therefore confirmed as Val.His.Pro.Phe.

Peptide HC3. This contained histidine and leucine and the FDNB technique showed histidine to be *N*-terminal. This helped to confirm the *C*-terminal sequence of hypertensin, previously shown to be Phe.His.Leu by other techniques.

Consideration of all these results showed that the amino acid sequence in hypertensin was

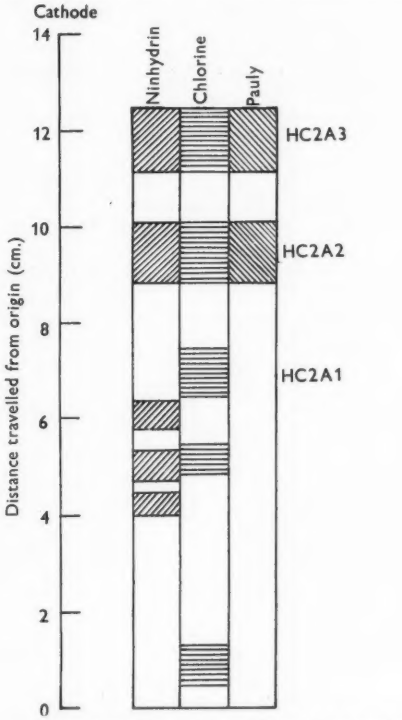

Fig. 5. Result of electrophoresis, for 1·25 hr. in 2N acetic acid at 15 v/cm., of a partial hydrolysate of peptide HC2. Bands stained by various techniques. Scale, ×4 in horizontal direction.

Table 5. *Peptides isolated by electrophoresis of a partial acid hydrolysate of HC2*

Peptide	Amino acids present	*N*-Terminal residue	Structure
HC2A1	Pro, Phe	Not determined	Pro.Phe
HC2A2	His, Pro, Val, Phe	Not determined	Unchanged HC2
HC2A3	His, Val	Val	Val.His

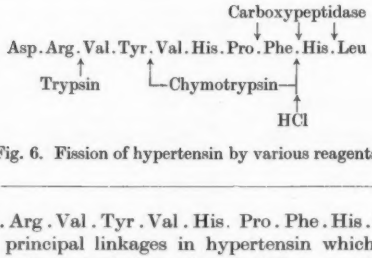

Fig. 6. Fission of hypertensin by various reagents.

Asp . Arg . Val . Tyr . Val . His . Pro . Phe . His . Leu. The principal linkages in hypertensin which are broken by the techniques described above are shown in Fig. 6.

DISCUSSION

N-Terminal sequence in hypertensin. When this work was commenced it was not certain that hypertensin had an open-chain structure. Although it seemed probable that it possessed an *N*-terminal residue consisting of aspartic acid,* the yield of DNP-aspartic acid obtained on hydrolysis of DNP-hypertensin was not good. Furthermore, this was obtained only when bicarbonate was used in the reaction with FDNB; with trimethylamine, the yield was extremely small. On the other hand, a simple cyclic structure seemed unlikely when it was found that the molecule was attacked by carboxypeptidase in the presence of di*iso*propyl phosphorofluoridate. In the course of the structural studies described in this paper, the presence of an *N*-terminal residue of aspartic acid has been demonstrated in several ways. The Edman degradation revealed aspartic acid as the *N*-terminal residue and, furthermore, both chymotrypsin and trypsin digests contained peptides possessing *N*-terminal aspartic acid, the DNP derivative of which was obtained in good yield by the FDNB method with trimethylamine as buffer. It seems unlikely that both these enzymes could break the same bond in view of their widely different specificities, and it thus follows that the amino group of aspartic acid must have been free in the original hypertensin molecule. It was also found that aminopeptidase, which is known to require a free *N*-terminal residue in its peptide substrate (Smith, Spackman & Brown, 1955), rapidly attacked hypertensin with liberation of aspartic acid and all the remaining amino acids. Although the work of Schroeder & Le Gette (1953) has shown that aspartyl peptides may give rather poor yields in the FDNB method, it seems possible that the chemical reactivity of the amino group in hypertensin is hindered in some way. This is indicated by the marked difference in the yield obtained when trimethylamine was replaced by sodium bicarbonate. A low yield of the *N*-terminal serine residue of corticotrophin (ACTH) was obtained by Levy & Li (1955), using the FDNB technique; this was due to destruction during acid hydrolysis. Owing to the scarcity of material, this interesting point has not been examined further.

Considering in more detail the results of the Edman degradation of hypertensin, Fig. 1 shows that 4·2 micro-equivalents of alkali were consumed in the reaction with phenyl *iso*thiocyanate. Hypertensin prepared by the method of Peart (1956) and freed from inorganic impurities as described in this paper could contain a maximum of four molecules of bound hydrochloric acid. Of these, the two molecules bound to the histidine residues would be completely titrated at pH 9, whereas the one bound to arginine would not. The two carboxyl groups would also be completely titrated. Thus a maximum of four titratable groups would be present and in addition one molecule of alkali would be consumed in the reaction:

$$R.NH_3^+ + PhN{:}C{:}S + OH^- \rightarrow R.NH.CS.NH.Ph + H_2O.$$

The molecular weight of a tetrahydrochloride of hypertensin would be 1427. The weight of hypertensin actually taken, 1·2 mg., should therefore consume $(1{\cdot}2/1{\cdot}43) \times 5 = 4{\cdot}2$ micro-equivalents. This consumption of the maximum possible quantity of alkali is satisfactory evidence that hypertensin contains one free amino group per ten amino acid residues. The change that occurred in the absorption when the phenylthiocarbamyl derivative of hypertensin was treated with 3N hydrochloric acid (Fig. 2) is characteristic of the formation of a phenylthiohydantoin derivative of the *N*-terminal amino acid (Fraenkel-Conrat *et al.* 1955). With the value of 16 000 for the molecular extinction coefficient (Fraenkel-Conrat *et al.* 1955) the yield of phenylthiohydantoin was calculated to be 55·9%. The fact that this characteristic change in the

* In this discussion aspartic acid is used in a somewhat vague sense. None of the techniques used, with the exception of aminopeptidase digestion, would have distinguished between aspartic acid and asparagine.

absorption spectrum was not observed in the subsequent stages is believed to be due to the accumulation of artifacts which absorb light in the region of minimum absorption of the thiohydantoin. The curve would thus be flattened out. It seemed unlikely that the absorption at 265 mμ. represented a true measure of the yields of the thiohydantoins in these cases and no attempt was made to calculate them. The idea that artifacts accumulated was upheld by the results of alkali titration in the reactions with phenyl *iso*thiocyanate; the uptake was always greater than theoretical from step 2 onwards. Fortunately the constant end slope, signifying the end of the reaction, was still clearly visible on the graphs plotted during these experiments.

Although it was found possible to identify thiohydantoins of amino acids by the various techniques which have been described (see Fraenkel-Conrat *et al.* 1955) these were not sufficiently reproducible to warrant their use with hypertensin. Various methods of hydrolysis of the thiohydantoins to amino acids have been investigated (see Fraenkel-Conrat *et al.* 1955); most recently Schramm, Braunitzer & Schneider (1955) have used hydriodic acid for the hydrolysis of the thiohydantoin derivative of proline. It was found that this method gave better results than other methods with the thiohydantoins of all the amino acids present in hypertensin. The thiohydantoin derivative of arginine was not investigated. This method was therefore used for the identification of the thiohydantoins produced during the Edman degradation of hypertensin and peptide HC2. It was found that all chromatograms contained ninhydrin-positive artifact spots, often as strong as the *N*-terminal amino acid in the peptide; these were not a result of the method of hydrolysis of the thiohydantoins, which had already been found to give amino acids substantially free from other substances. Fortunately, these artifact spots were produced consistently in every step of the degradation and the R_F values were different from the amino acids in hypertensin. It is evident from a recent preliminary publication (Edman, 1956) that the cleavage of phenylthiocarbamyl derivatives of peptides is more complicated than was previously believed, but until a full account appears it is not possible to decide whether the difficulties encountered in the present work could be explained in the light of this knowledge.

In view of the difficulty which was sometimes experienced in obtaining a satisfactory yield of tyrosine on acid hydrolysis of hypertensin, it was interesting to find similar results on hydrolysis of peptide HC1 (Table 3). The tyrosine appeared to be present in only one-half of the theoretical amount, but it was obtained in good yield when liberated by long digestion with carboxypeptidase (Table 4). Although tyrosine has been shown to be one of the less-stable amino acids (Hirs, Stein & Moore, 1954), destruction to this extent is abnormal and is perhaps due to the presence of carbohydrate extracted from the filter paper or to oxidation, in view of the very small quantities of material being handled.

All the amino acids present in hypertensin could be assumed to have the L-configuration since they were freely liberated by aminopeptidase (see Smith *et al.* 1955, for evidence of optical specificity).

SUMMARY

1. The amino acid sequence in a hypertensin is shown to be aspartic acid.arginine.valine.tyrosine.valine.histidine.proline.phenylalanine.histidine.leucine.

2. On the basis of digestion by aminopeptidase, it is believed that all the amino acids have the L-configuration and that the *N*-terminal residue is aspartic acid, not asparagine.

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Steroid Utilization and Fatty Acid Synthesis by the Larva of the Housefly *Musca vicina* Macq.

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Recent studies on the lipid requirements (Silverman & Levinson, 1954) of, and on the specificity of steroids as growth factors (Bergmann & Levinson, 1954) for, housefly larvae, have shown that a sterol (phytosterol or zoosterol) is the only lipid essential for growth and metamorphosis. The growing larva consumes the sterols of the wheat bran of the medium together with considerable amounts of unsaturated fatty acids; however, whilst the latter are metabolized during pupation, the sterol content remains relatively constant throughout this period (Levinson & Silverman, 1954). The importance of steroids in insect physiology has been known for some time, but relatively little work appears to have been done on the housefly.

In the present study, therefore, the utilization of different steroids by *Musca vicina* larvae has been investigated. A preliminary account of this work has been given by Levinson & Bergmann (1955).

MATERIALS AND METHODS

Musca vicina *Macq.* The strain of houseflies was the same as that used in previous investigations; the method of its maintenance has been described by Silverman & Silverman (1953).

Preparation of newly hatched larvae. Adult houseflies, 2 days of age, were fed for 2 days on a water suspension of 20% (w/v) powdered milk and subsequently sucrose and water. Six- to seven-day-old females were allowed to oviposit within 3 hr. on a pad of damp cotton wool. Approximately 750 eggs were collected, carefully washed with water, placed on damp blotting paper in a covered Petri dish (diam. 9 cm.) and incubated at 30°. Usually 80–90% of these eggs were fertile, and larvae hatched from them $11 \pm 1{\cdot}5$ hr. after oviposition. Soon after hatching, the larvae congregated inside the cover of the Petri dish.

Since up to the present time no satisfactory method for the sterile culture of houseflies is known, the experiments have been carried out in the presence of a controlled bacterial flora. Although this flora failed to provide utilizable steroids for larvae reared on a lipid-free diet, it is possible that the bacteria cause changes in the steroid structure before its ingestion.

Rearing method. The medium (approximately 1 g.) was placed in a test tube (150 mm. × 15 mm.), and 0·15 ml. of a suspension of stock bacterial flora (Silverman & Silverman, 1953; $1{\cdot}5$–$2{\cdot}5 \times 10^8$ bacteria/ml., 70% of which were *Escherichia coli*, the balance *Sarcina*, *Lactobacillus* and *Bacillus subtilis*) was added together with one larva. The tubes were then incubated at 35°. The internal temperature of the medium was $36 \pm 0{\cdot}5°$ during the period of larval growth. At an age of 72 hr., the larvae were removed from their diets in the same sequence as they had been inoculated, cleaned of adhering medium, and weighed to the nearest 0·1 mg. Immediately after weighing, they were returned to their media. For convenient observation of the appearance of pupae, a flock of cotton wool was loosely plugged into each test tube (Fig. 1). The larvae were reincubated (35°) and inspected daily at a constant time until pupation or death. Records were kept of the number and average weight of the larvae, as well as of the number of larvae pupating or perishing on successive days and the number of adults emerging from the pupae. In a few instances the fecundity of the females was determined.

Composition of the experimental media. No. 1. Wheat-bran diet: a quantity (25 g.) of sieved wheat bran (Golan-Flour Mills, Tel-Aviv) was covered with 50 ml. of pure ether and incubated at 35° for 2 days, at which time the ether had completely evaporated. Before use, 25 ml. of water was mixed uniformly with the bran. The ether treatment was applied in order to make the conditions as similar as possible for all three diets used.

No. 2. Casein diet: the chemicals listed in Table 1 were intimately mixed and the pH was adjusted to 7·0 by dropwise addition of 5N-NaOH solution. Pieces of chloroform-

extracted cotton wool, weighing about 0·1 g., were soaked with portions of 1 g. of the resulting mixture. The vitamin mixture in this diet was selected in conformity with the vitamin content of several yeast species (Dunn, 1952). The large excess of cholesterol in this diet was applied in order to ensure that each larva would receive an adequate quantity of this compound.

No. 3. Steroid-free diet: a quantity (500 g.) of sieved wheat bran was extracted at least 20 times with 1·5 l. of $CHCl_3$ in a Soxhlet apparatus. These conditions were found to be sufficient to free the wheat bran from steroids. It should be mentioned here that the procedure formerly employed for the preparation of a basic lipid-free diet (Silverman & Levinson, 1954) was found to yield media which, in spite of their negative Liebermann–Burchardt reaction, still promoted some larval growth. An amount (25 g.) of the steroid-free material was mixed with 25 ml. of a 5% (w/v) solution of dried-yeast extract (Baltimore Biological Laboratories, U.S.A.) in sterile water. On this diet, newly hatched larvae attained an average weight of 1·0 mg., but died at an age of 2–3 days.

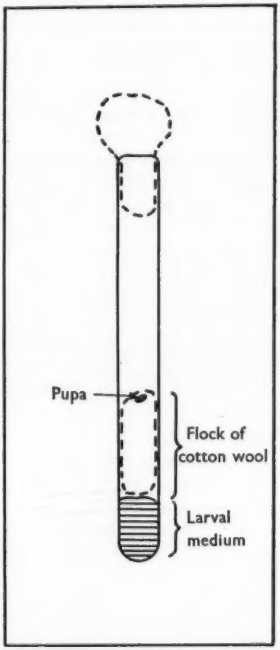

Fig. 1. Method of rearing single fly larvae. A flock of cotton wool plugged loosely above the larval medium constitutes the site chosen by the larva for pupation. The contrast of the colours of the two layers facilitates the observation of the appearance of pupae and adults inside the cotton wool.

Steroids. The following materials were commercial preparations: cholesterol, β-sitosterol (24b-ethylcholest-5-en-3β-ol), stigmasterol, ergosterol, calciferol, 7-hydroxycholesterol, cholesteryl acetate, cholic acid, deoxycholic acid. oestrone, dehydro*epi*androsterone acetate, 3β-hydroxy-5β-androstan-17-one acetate, strophanthidin, digitonin, saponin 'white'.

A number of steroids the authors owe to the courtesy of various donors: cholesterol, purified via the dibromide (Professor L. F. Fieser, Harvard University); 'sitosterol' and 2α-chlorocholestan-3β-ol (see Beerenboom, Djerassi, Ginsburg & Fieser, 1953) (Professor D. Ginsburg, Hebrew Technical College, Haifa); γ-sitosterol (24*a*-ethylcholest-5-en-3β-ol) (Dr W. Graham, Organon Laboratories Ltd., England); diosgenin (Lepetit, Milano, Italy).

The remaining steroids have been prepared according to the references indicated in parentheses: Cholesteryl laurate, m.p. 90° (Page & Rudy, 1930). Cholesteryl chloride (3β-chlorocholest-5-ene), m.p. 96° (Diels & Blumberg, 1911). Cholesterylaniline, m.p. 189° (Lieb, Winkelmann & Koeppl, 1934). Cholest-4-en-3β-ol (*allo*cholesterol), m.p. 132° (Windaus, 1927). Wheat-bran sterols (Silverman & Levinson, 1954). Cholestan-3α-ol (*epi*cholestanol), m.p. 181–182° (Ruzicka, Brüngger, Eichenberger & Meyer, 1934). Cholestan-3β-ol acetate, m.p. 111° (Willstätter & Mayer, 1908). 3β-Acetamidocholest-5-ene, m.p. 244° (Windaus & Adamla, 1911). Cholest-5-en-3-one, m.p. 127° (Butenandt & Schmidt-Thome, 1936). 6-Bromocholest-4-en-3-one, m.p. 130–131° (Ruzicka, Bosshard, Fischer & Wirz, 1936; Dane, Wang & Schulte, 1937). 3-Acetoxycholesta-2:4-diene (enol acetate of cholest-4-en-3-one), m.p. 80° (Ruzicka & Fischer, 1936). Cholestan-3-one, m.p. 128–129° (Diels & Abderhalden, 1906). 7-Oxocholesteryl acetate, m.p. 154° (Windaus, Lettré & Schenck, 1935). 7-Oxocholesteryl chloride, m.p. 145° (Marker, Kamm, Fleming, Popkin & Wittle, 1937). 7-Dehydrocholesterol (cholesta-5:7-dien-3β-ol), m.p. 144° (Windaus *et al.* 1935). Cholest-5-ene, m.p. 90° (Mauthner & Suida, 1894). Dehydrocholic acid (3:7:12-trioxocholanic acid), m.p. 237° (Hammarsten, 1881). 17β-Hydroxy-5α-3-androstanone acetate, m.p. 151° (Butenandt, Tscherning & Hanisch, 1935). *iso*Strophanthidin, m.p. 274–276° (Windaus & Herrmanns, 1915).

Table 1. *Composition of casein diet* (*diet no.* 2)

	g.		mg.
Chloroform-extracted casein (vitamin-free, Hoffman-La Roche)	3·750	Thiamine chloride	0·250
		Riboflavin	0·500
Salt mixture no. 2 (U.S.P. XII)	0·150	Nicotinic acid	2·500
L-Cystine	0·100	Pantothenic acid	0·500
Cholesterol, purified via the dibromide	0·050	Folic acid	0·250
Choline chloride	0·025	Pyridoxine	0·250
Inositol	0·010	Biotin	0·0025
Nipagin M	0·150	*p*-Aminobenzoic acid	0·500
		Vitamin B_{12}	0·001

Water up to 50 g.

In order to test the utilization or antagonist action of the steroids, they were dissolved in 50 ml. of ether, the solution was poured on 25 g. of the dry diets nos. 1 and 3, and the solvent (which was just sufficient to cover the media) evaporated at 35°, leaving a crystalline residue as evenly distributed as possible. The bile acids were tested as sodium salts, being added to the liquid components of the diets. As a rule, the liquid components were admixed with the dry diets just before the start of a series of experiments.

Determination of lipid content. For the determination of lipid content, larvae were reared at a density of two individuals/g. of diet (nos. 1–3, Table 2) at a temperature of 35°. Approximately 25 g. of larvae was collected from each experimental diet and analysed in batches of 5–10 g. The larval age at the time of analysis is indicated in Table 2. The larvae were desiccated, powdered and extracted exhaustively with ether, and the ether residue was saponified with 5N ethanolic KOH solution. The fatty acids were liberated by adding an excess of H_2SO_4, extracted with light petroleum (boiling range 40–60°) and titrated with 0·1N-KOH, with phenolphthalein as indicator. The resulting soap was dried and weighed. The amount of saponifiable fat was computed according to Wizoeff (1927) and is recorded as percentage of live weight in Table 2.

Inhibition of mould growth in the wheat-bran media. The wheat-bran diet, at a rearing temperature of 35°, would become infested with certain moulds which might provide the larvae with an additional supply of steroids. It was necessary, therefore, to add to the diet 0·3% of Nipagin M (methyl *p*-hydroxybenzoate), an antiseptic active against a wide range of moulds and bacteria (Aalto, Firman & Rigler, 1953), but apparently without effect on growing housefly larvae.

RESULTS

Table 2 shows that growing housefly larvae are able to synthesize body fat from diets consisting largely of carbohydrates (no. 3) or proteins (no. 2). The rate at which body fat is synthesized in these cases appears to be similar to that at which it is taken up from a fat-containing diet. The characterization of the fatty acids synthesized is reserved for a later publication.

The larvae grown on the fat-free diets developed into viable adults which reproduced normally when fed on sterile solutions of fat-free peptone and sucrose. The average number of eggs laid per female and life time (temperature, $30 \pm 1°$; relative humidity, $50 \pm 5\%$) amounted to 300 ± 50, their fertility being similar to that of the controls. Under these conditions, the average length of life was 20 ± 1 days.

In order to ascertain whether the presence of such large amounts of sterols as contained in the wheat-bran diet (no. 1) is necessary for larval growth and metamorphosis, the experiments summarized in Table 3 have been carried out with diet no. 3. Each experiment represents a total of 100 larvae, employed in four parallel batches. The variation between such batches did not exceed 10%. Newly hatched larvae, which did not receive cholesterol, were able to gain 0·9 mg./larva, but died at an age of 2–3 days. Addition of 2 μg. of cholesterol/g. of diet

Table 2. *Synthesis of body fat by housefly larvae*

	Diet				Larvae		
	Lipids contained in diet (% of wet wt.)		Lipids added to diet (% of wet wt.)				
Diet no.	Total lipid	Sterol	Total lipid	Cholesterol	Age at time of analysis (hr.)	Average body wt. (mg.)	Average lipid content (% of live wt.)
1. Wheat bran	1·8	0·1	0·0	0·0	72	23·8	4·9
2. Casein	0·0	0·0	0·1	0·1	72	22·7	4·5
3. Steroid-free	0·0	0·0	0·1	0·1	48	18·7	3·8

Table 3. *Influence of increasing amounts of dietary cholesterol on survival, growth rate, pupation and emergence of the housefly*

Cholesterol added to diet no. 3 (μg./g.)	Larvae surviving after 72 hr. (%)	Average larval wt. at age 72 hr. (mg.)	Larvae pupating (%)	Percentage of pupation at subsequent age (hr.)					Larvae developing into adults (%)
				96	120	144	168	192	
0	4*	1·0	0	—	—	—	—	—	—
2	16	14·0	12	—	—	16	50	34	4
4	18	16·1	16	—	—	36	64	—	12
6	28	17·7	28	—	57	43	—	—	24
8	36	19·0	36	—	64	36	—	—	32
12	48	20·1	44	12	66	22	—	—	36
14	51	21·5	50	36	54	10	—	—	44
18	64	22·3	60	60	40	—	—	—	57
30	80	22·6	68	80	20	—	—	—	63
36	84	22·5	84	78	22	—	—	—	78
50	96	22·8	96	83	17	—	—	—	88

* Died within the following 3 hr.

Table 4. *Effect of steroids and certain other substances on housefly larvae*

Steroids were added to the steroid-free diet (no. 3) in a concentration of 0·1%.

Expt. no.	Steroid added	Average larval wt. at age 72 hr. (mg.)	Larvae pupating (%)	Mean time to pupation (days)	Larvae surviving at 72 hr. (%)
1	Cholesterol	24·2	96	4·1	—
2	Cholesterol, purified via dibromide	24·6	98	4·0	—
3	Cholest-4-en-3β-ol	25·0	74	4·0	—
4	Cholesteryl acetate	24·0	95	4·0	—
5	Cholesteryl laurate	24·0	88	4·0	—
6	Wheat-bran sterols	23·9	82	4·5	—
7	'β'-Sitosterol	22·8	53	5·5	—
8	'γ'-Sitosterol	21·9	52	5·5	—
9	Enol acetate of cholest-4-en-3-one	19·5	59	5·0	—
10	7-Dehydrocholesterol	11·1	44	6·3	—
11	Ergosterol	19·0	39	6·4	—
12	Stigmasterol	18·1	28	6·8	—
13	Calciferol	3·5	0	—	44*
14	Cholest-5-en-3-one	3·0	19	8·1	—
15	6-Bromocholest-4-en-3-one	4·7	16	6·2	22
16	7-Hydroxycholesterol	—	0	—	0
17	7-Oxocholesteryl acetate	3·4	4	7·6	28
18	7-Oxocholesteryl chloride	—	0	—	0
19	Cholesteryl chloride	—	0	—	0
20	Cholesterylaniline	1·1	0	—	18
21	Cholest-5-ene	—	0	—	0
22	Dehydro*epi*androsterone acetate	—	0	—	0
23	3β-Hydroxy-5β-androstan-17-one acetate	1·3	0	—	4
24	Diosgenin	3·0	4	8·0	27
25	Cholestan-3β-ol acetate	7·0	0	—	68*
26	Cholestan-3-one	2·6	0	—	16*
27	2α-Chlorocholestan-3β-ol	—	0	—	0
28	3β-Acetamidocholest-5-ene	—	0	—	0
29	Cholestan-3α-ol	—	0	—	6*
30	17β-Acetoxy-5α-androstan-3-one	1·1	0	—	10
31	Oestrone	—	0	—	0
32	Cholic acid	1·5	0	—	6
33	Deoxycholic acid	1·3	0	—	4
34	Dehydrocholic acid	1·0	0	—	2
35	Saponin	—	0	—	0
36	Digitonin	—	0	—	0
37	Strophanthidin	—	0	—	0
38	*iso*Strophanthidin	—	0	—	0

* Died within the following 24 hr.

no. 3 fed to one larva permits an average weight gain of 13·9 mg., but this level is insufficient by far to promote normal growth and pupation. The larval weights increase proportionally with increasing concentrations of dietary cholesterol up to a level of 30 μg./g. of diet, which is, however, not optimum for larval survival, pupation and adult emergence. The level of 50 μg. of cholesterol/g. of diet was found to be the minimum concentration required for normal growth and metamorphosis of *Musca vicina*. Addition of very large amounts of cholesterol to the diet, e.g. 150 mg./g. of diet, did not affect its nutritional value for the larvae. The amount of cholesterol actually ingested by each larva is unknown.

The study of the utilization of thirty-eight steroids fed to housefly larvae at a concentration of 0·1% of the diet no. 3 is summarized in Table 4. Values of less than 20% of the larvae reaching pupation are considered as indicating 'no utilization'; such values are probably due to contamination of the substances tested with traces of utilizable steroids.

Some of the steroids listed in Table 4 were not only non-utilizable for the development of the larvae, but inhibited the utilization of the wheat-bran sterols present in the diet. It can be seen (Table 5) that cholesteryl chloride and 7-oxocholesteryl chloride suppressed larval growth and pupation. Cholestan-3-one and cholestan-3α-ol were even more active in this respect than 7-oxocholesteryl chloride. Digitonin decreased growth and the rate of pupation, proportionally to its concentration. It may be assumed that the latter compound is inhibitory because it can precipitate steroids possessing a 3β-hydroxyl group. Since

Table 5. *Inhibitory effect of certain steroids and digitonin on the growth and pupation of larvae*

Steroid added to wheat-bran diet	Concn., % of wet diet	Average larval wt. at age 72 hr. (mg.)	Larvae pupating (%)
—	—	23·8	80
7-Oxocholesteryl chloride	0·20	20·6	42
Cholesteryl chloride	0·10	13·3	30
Cholestan-3α-ol	0·20	9·0	3
Cholestan-3-one	0·20	7·6	2
Digitonin	0·20	—	0
Digitonin	0·17	—	0
Digitonin	0·10	15·2	29
Digitonin	0·05	23·0	55
Digitonin	0·02	24·0	82

Table 6. *Inhibitory effect of cholesteryl chloride on the growth and pupation of larvae*

Cholesterol in diet (%)	Cholesteryl chloride in diet (%)	Ratio	Average larval wt. at age 72 hr. (mg.)	Larvae pupating (%)
0·2	—	—	24·0	93
—	0·1	—	—	0
0·2	0·1	2:1	25·9	97
0·1	0·1	1:1	24·2	90
0·05	0·1	1:2	19·3	88
0·03	0·1	1:3	17·8	84
0·005	0·1	1:20	12·7	40*
0·001	0·1	1:100	12·4	16*
0·001	—	—	19·8	40
0·0002	0·1	1:500	5·0	0
0·0002	—	—	13·2	10

* These pupae did not develop into adult houseflies.

cholesteryl chloride has been found to inhibit the utilization of cholesterol by growing *Attagenus* (McKennis, 1947, 1954) and *Blattella* (Noland, 1952, 1954*b*), an attempt was made to establish the existence of such an antagonism for the housefly larva. Table 6 shows that larvae fed on cholesterol and cholesteryl chloride at a ratio of 1:1 grew and pupated normally; decreasing the concentration of cholesterol at a constant level of cholesteryl chloride in the diet suppressed progressively larval growth and pupation. Thus a ratio of 1:20 of cholesterol and cholesteryl chloride permitted only 40% pupation and prevented the emergence of adult flies.

DISCUSSION

It has been shown that not only are housefly larvae able to synthesize their body fat from diets consisting largely of carbohydrates or proteins, but also that they do not require dietary carbohydrate, as they can subsist on a casein diet (diet no. 2). In this respect they differ from a number of phytophagous larvae (Fraenkel & Blewett, 1943; Lafon & Teissier, 1939).

Furthermore, the housefly does not require the unsaturated fatty acids which are essential for growth and wing formation in *Ephestia* (Fraenkel & Blewett, 1947) and for the metamorphosis and reproduction in *Loxostege* (Pepper & Hastings, 1943). Among the flies, *Phaenicia sericata* needs neither fat nor carbohydrate in the diet (Hobson, 1935), whereas the parasitic *Pseudosarcophaga affinis* requires both for optimum growth (House, 1954). On the other hand, the housefly is incapable of steroid synthesis.

From a quantitative aspect, the requirements of the housefly larvae for cholesterol appear to be similar to those of two other dipterous larvae, *Phaenicia sericata* (Hobson, 1935) and *Drosophila melanogaster* (Van't Hoog, 1936). It was surprising to find that *Musca vicina* tolerates as much as 15% of dietary cholesterol, since the presence of 6·3% of cholesterol in the diet had been found to be fatal to *Callosobruchus chinensis* (Ishii, 1951).

The experiments summarized in Table 4 show that the effect of the steroids depends on their molecular structure. The following modifications of the structure of cholesterol do not impede utilization: (1) Esterification of the 3-hydroxyl group with fatty acids, since cholesterol can be replaced by its acetate or laurate. This may be due to the ability of the larva to split these esters enzymically. (2) Presence of a second double bond in the polycyclic system or of a double bond in the side chain, since 3-acetoxy-cholesta-2:4-diene, 7-dehydrocholesterol, ergosterol and stigmasterol are utilized.

(3) Branching of the side chain, since 'β'- and 'γ'-sitosterol are utilizable. It is worthy of note that the natural mixture of wheat-bran sterols is better utilized than either 'β'- or 'γ'-sitosterol. (4) Presence of the double bond at 4:5 instead of at 5:6 as in cholest-4-en-3β-ol.

On the other hand, there exist certain modifications of the molecule which result in loss of utilizability. These are: (1) Absence of the hydroxyl group in position 3 (cholest-5-ene). (2) Introduction of a hydroxyl or keto group in the 7 position. (3) Substitution of the hydroxyl group of cholesterol by the anilino or acetamido group or by chlorine. (4) Removal of the side chain (acetates of dehydro*epi*androsterone and of 3β-hydroxy-5β-androstan-17-one and oestrone) or its replacement by the spiroketal structure (diosgenin). (5) Aromatization of ring *A* together with removal of the side chain (oestrone). (6) Conversion of the cholesterol into cholest-5-en-3-one or 6-bromocholest-4-en-3-one. (7) Saturation of the $\Delta^{5:6}$ double bond (cholestan-3β-yl acetate, cholestan-3-one, 2α-chlorocholestan-3β-ol). (8) Epimerization of the C_3 hydroxyl group (cholestan-3α-ol). (9) The more radical changes of the total structures expressed by the formulae of bile acids, strophanthidin, saponins and calciferol.

Recently, similar observations have been made in the protozoan *Paramecium aurelia*. A sterol (other than cholesterol or ergosterol) is required as growth factor, and it can be replaced by a steroid ketone (carbonyl in 3 position). However, the presence of more than one double bond in the ring or the more drastic changes required for the formation of cholic acid, sex hormones or steroid sapogenins destroy the growth-promoting activity (Conner, van Wagtendonk & Miller, 1953; Conner & van Wagtendonk, 1955). Similar investigations have been carried out before for other micro-organisms, namely, *Trichomonas columbae* (Cailleau, 1937), *Pleuropneumonia*-like organisms (Edward & Fitzgerald, 1951), *Labyrinthula vitellina* (Vishniac & Watson, 1953; Vishniac, 1955) and, recently, *Saccharomyces cerevisiae* and *Escherichia coli* (Adelberg, Roslansky, Myers & Coughlin, 1955).

Steroid utilization differs amongst insect species. So far about fifteen species, belonging to the orders Coleoptera, Lepidoptera, Orthoptera and Diptera, have been studied. They were all found capable of utilizing fatty acid esters of cholesterol, and Noland (1954*b*) has assumed that cholesterol absorption requires an esterase (ester-synthesizing enzyme). However, cholesteryl trimethylacetate, which is rather resistant to enzymic hydrolysis, was reported not to be utilizable by *Blattella* (Noland, 1954*a*).

Utilization of steroids containing more than one double bond differs among various species. The enol acetate of cholest-4-en-3-one (3-acetoxycholesta-2:4-diene) is utilized by *Musca* and by *Drosophila* (Van't Hoog, 1936), but not by *Aedes* (Goldberg & De Meillon, 1948) and *Blattella* (Noland, 1954*a*). For the rat, cholestenone is not equivalent to cholesterol since the rat cannot reduce the unsaturated ketone to the sterol (Stokes, Fish & Hickey, 1955). 7-Dehydrocholesterol was found to be well utilized by the carnivorous *Dermestes* (Fraenkel, Reid & Blewett, 1941) and by the phytophagous beetles *Lasioderma*, *Ptinus*, *Silvanus*, *Sitodrepa* and *Tribolium*, and the moth *Ephestia kuehniella* (Fraenkel & Blewett, 1943), but it was less available to the zoophagous carpet beetle *Attagenus* (McKennis, 1947) and to the mainly coprophagous *Musca*. Among the phytophagous species, *Drosophila*, feeding mainly on living yeast, and the six above-mentioned species utilize ergosterol as well as cholesterol, and the zoophagous Dermestidae do not utilize it at all. *Phaenicia* (Hobson, 1935), *Aedes*, *Blattella* and *Musca*, which feed both on animal and vegetable matter, appear intermediate between phytophagous and zoophagous species: they utilize ergosterol, but poorly. *Callosbruchus* (Ishii, 1951), feeding preferably on leguminous seeds, utilizes stigmasterol well, whereas *Aedes*, *Drosophila*, *Blattella* and *Musca* utilize it less. The sitosterols are best utilized by the six flour insects, but they are less utilizable by *Drosophila*, *Blattella* and *Musca*, which feed only occasionally on cereals. However, the carnivorous *Dermestes* is unable to utilize sitosterols. All phytophagous species thus far investigated are able to utilize cholestan-3β-ol. *Musca*, however, resembles the carnivorous *Dermestes* and *Attagenus* in being unable to utilize this compound. It may, therefore, be concluded that steroid utilization of the species studied is regulated by their feeding habits. The smallness of the structural changes which affect steroid utilization by insects suggest that the specificity of these growth-promoting substances is rather high. In this respect it should be recalled that the oestrone-induced uterine growth is also antagonized by steroids similar to oestrone (Huggins & Jensen, 1955), that the hydroxysteroid dehydrogenases of *Pseudomonas* are inhibited by other steroids (Talalay & Marcus, 1954), and that 3β-cholestanol prevents the increase of plasma cholesterol in chicken reared on a high-cholesterol diet (Siperstein, Nichols & Chaikoff, 1953).

SUMMARY

1. Housefly larvae are able to synthesize body fat from dietary protein; they require neither fatty acids nor carbohydrate in the medium. Furthermore, they reproduce normally without any dietary source of fat during larval and adult life.

2. Cholesterol in quantities of 2 μg./g. of diet stimulates growth of housefly larvae. Larval growth

is proportional to the concentration of dietary cholesterol, up to a certain limit. Excess of cholesterol in the diet has no adverse effect.

3. The influence of steroid structure on the utilizability has been studied. It has been found that some steroids are not only not utilized, but they inhibit the utilization of other steroids.

4. Cholesterol utilization by housefly larvae is reversibly inhibited by cholesteryl chloride.

5. The utilization of steroids by insects is discussed in connexion with their feeding habits.

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Preparation and Amino Acid Composition of Enzymically Dephosphorylated Casein

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Plimmer & Bayliss (1906) were the first to show that casein could be completely dephosphorylated by incubation at 37° with 1% sodium hydroxide for a period of 24 hr. Acidification of the digest after the completion of dephosphorylation precipitated a substance resembling casein. Rimington (1927) analysed the dephosphorylated protein and noticed little difference in composition from that of casein except in amide and arginine nitrogen. The protein, termed dephosphorized caseinogen, gave all the colour reactions of casein. Plimmer & Lawton (1939) called attention to the fact that the action of alkali on casein was not as simple as appeared from the experiments of Rimington. These workers observed a splitting up of the casein molecule by alkali, resulting in the formation of the protein termed dephosphorized caseinogen by Rimington and a proteose precipitable by half saturation with ammonium sulphate. The dephosphocasein which accounted for about 52% of the casein nitrogen and the dephosphocaseose which accounted for nearly a third of casein nitrogen had more or less the same amino acid composition as casein (Macara & Plimmer, 1940).

Experiments carried out in this Laboratory on the action of alkali on proteins indicate that, besides casein, other proteins are also degraded by prolonged incubation with alkali, resulting in the liberation of a significant amount of acid-soluble nitrogen (Sundararajan, 1956). Further, the racemization of amino acids and the hydrolysis of amide linkages in the protein brought about by the action of alkali render this reagent unsuitable for the study of the dephosphorylation of proteins.

Enzymic methods have been used in recent years in studies of this type. Thus the use of citrus-fruit phosphatase by Mecham & Olcott (1949) for bringing about the dephosphorylation of phosvitin and the preparation of dephosphorylated ovalbumin by Perlmann (1952) through the use of an intestinal phosphatase preparation are typical of this new type of approach. The discovery of phosphoprotein phosphatase by Harris (1946) provided the protein chemist with a new and far more effective tool for bringing about modification of proteins.

The availability of purified preparation of phosphoprotein phosphatase from ox spleen (Sundararajan & Sarma, 1954*a*) has led in the present investigation to a study of its usefulness as a specific dephosphorylating agent for casein. In preliminary experiments it was observed that casein solutions, incubated with purified preparations of the enzyme at pH 6·0, developed a milkiness, followed by the gradual formation of a precipitate within a few hours. After about 24 hr. the precipitate had settled down at the bottom of the tube, leaving a clear supernatant. Later these investigations were repeated with larger portions of casein and the precipitated protein was separated and estimated for its phosphorus content. The precipitate was consistently found to be free from phosphorus (Sundararajan & Sarma, 1954*b*). Since the enzyme preparations used to bring about dephosphorylation should be free from proteolytic activity so as to avoid undesirable side reactions, attention was next devoted to the preparation of protein phosphatase devoid of proteolytic activity. An effective method was to heat the enzyme preparation to 80° for 5 min. This treatment brought about selective inactivation of the proteolytic activity, leaving the phosphatase activity unaffected. The present paper gives details of this procedure and of the preparation of dephosphorylated casein through the use of this enzyme. The amino acid make-up of the dephosphorylated protein and the changes accompanying the enzymic dephosphorylation of casein have also been investigated.

METHODS

Measurement of phosphatase activity. Methods for the determination of phosphatase activity and definition of unit of enzyme activity are as described in a previous paper (Sundararajan & Sarma, 1954*a*).

Measurement of proteolytic activity. Proteolytic activity was determined by estimation of nitrogen in the trichloroacetic acid filtrate from enzyme digests according to the method of Koch & McMeekin (1924).

Analyses of proteins. Analyses of casein and dephosphorylated casein for the various constituents were carried out simultaneously and under identical conditions so as to make strict comparison of values possible. All analyses were carried out on air-dried protein samples. Moisture determinations were made as suggested by Chibnall, Rees & Williams (1943). Total nitrogen was determined according to the micro-Kjeldahl method of Chibnall *et al.* (1943), the

ammonia being estimated by distillation into boric acid (Ma & Zuazaga, 1942). Phosphorus determinations were carried out by the method of Fiske & Subbarow (1925) after digestion with sulphuric acid.

Amino acid analyses. Hydrolysis of the protein was carried out in 20% (w/w) hydrochloric acid at 110° for 24 hr. The acid was removed by repeated distillations *in vacuo* and the hydrolysate made up to a suitable volume.

Tyrosine and tryptophan estimations were carried out on alkali hydrolysates of the protein according to the methods of Bernhart (1938) and Horn & Jones (1945) respectively.

Cystine was determined according to the procedure of Kassell & Brand (1938) and methionine by the method of Horn, Jones & Blum (1946).

Arginine and histidine were estimated after a preliminary separation of these amino acids from the protein hydrolysate by precipitation with phosphotungstic acid (Van Slyke, Hiller & MacFayden, 1941). The colorimetric procedure of Macpherson (1946) was then employed for the determination of these amino acids.

Hydroxyamino acids were estimated by periodate oxidation. Threonine was estimated titrimetrically as acetaldehyde after periodate oxidation (Rees, 1946). Serine was determined by oxidation with periodate, distillation of formaldehyde by the method of Boyd & Logan (1942), and estimation of formaldehyde photometrically with chromotropic acid according to the micromethod of Frisell, Meech & Mackenzie (1954).

Glycine was estimated according to the procedure of Alexander, Landwehr & Seligman (1945).

Glutamic acid was determined manometrically as carbon dioxide after enzymic decarboxylation with *Clostridium welchii* SR12 according to Meister, Sober & Tice (1951).

Microbiological assay methods were employed for the estimation of leucine, isoleucine, valine, proline, phenylalanine, lysine, alanine and aspartic acid. Alanine was determined according to Sauberlich & Baumann (1949) and aspartic acid by the procedure of Hac & Snell (1945). Analyses of the other amino acids were carried out essentially according to the procedure outlined by Barton-Wright (1952).

RESULTS

Preparation of protease-free phosphoprotein phosphatase

Phosphoprotein phosphatase was purified from ox spleen according to the method described previously (Sundararajan & Sarma, 1954*a*). A minor modification in the original method was introduced in that the extraction of the enzyme from the tissue was carried out at pH 5·0 instead of at pH 5·8. The bulk of the inactive protein material was rendered insoluble by this procedure and sharp separation of the enzyme in the ammonium sulphate fractionation step was obtained. An outline of the modified procedure along with the purification obtained is given in Table 1. The purified preparation exhibited some amount of proteolytic activity on testing with casein and egg albumin as substrates. Heat denaturation was found, however, to be quite effective for the elimination of this activity. For this purpose, the enzyme solution, buffered at pH 5·0, was heated in a water bath maintained at 80°. At the end of 5 min. the solution was quickly cooled to room temperature and filtered. This treatment completely destroyed the proteolytic activity, leaving the phosphatase activity relatively unaffected. The amounts of inorganic phosphorus and acid-soluble nitrogen released from casein by such preparation during a 24 hr. incubation period are indicated in Table 2. It will be seen that a small amount of acid-soluble nitrogen is formed even after the heat-treatment. No such liberation was found, however, with egg albumin as substrate. The nature of acid-soluble nitrogen released from casein has been characterized by paper chromatography and is discussed in a later section.

Table 1. *Purification of phosphoprotein phosphatase from ox spleen*

Stage	Volume (ml.)	Total activity (units)	Overall yield (%)	Specific activity*
1	2550	9775	—	0·5
2	1635	6810	70·0	1·4
3	65	5230	53·5	139·0
4	40	3307	33·8	773·0

* Activity units/mg. of protein nitrogen.

Summary of procedures

(1) Ox spleen was homogenized with 2·5 volumes of saline solution containing 0·2 M acetate buffer at pH 5·0. (2) The supernatant was obtained on centrifuging of homogenate. (3) The supernatant was fractionated with ammonium sulphate. The precipitate obtained at 40–80% saturation was separated and dialysed. The precipitate separating on dialysis was extracted with saline solution at pH 5·0 and filtered. (4) The filtrate from (3) was fractionated with acetone. The precipitate obtained at 50–66% (v/v) acetone at 0° was extracted with saline at pH 5·0 and filtered.

Table 2. *Selective heat denaturation of proteolytic activity*

Activities determined with 0·1 ml. of purified phosphoprotein phosphatase preparation with casein as substrate (1 ml. of 10% solution) and thioglycollic acid (0·001 M) as activator. Incubation time, 24 hr.

Enzyme	Inorganic phosphorus released (μg.)	Acid-soluble nitrogen formed (μg.)	Nitrogen solubilized (%)
Untreated preparation	470	1350·0	9·6
Preparation held for 5 min. in a water bath at 80°	454	307·5	2·2

Preparation of dephosphorylated casein

Casein (25 g. of Light White Soluble, British Drug Houses, Ltd.) was added in small portions to 800 ml. of water contained in a beaker, with continuous stirring. After the protein had gone into solution, 10 g. of Celite Analytical Filter-Aid (diatomaceous silica filter aid L665 obtained from Fisher Scientific Co., U.S.A.) were added, the suspension stirred well and filtered through a layer of Celite on a Büchner funnel. The clear filtrate was transferred to a 2 l. bottle. A molar solution of acetate buffer (pH 5·8, 50 ml.) was added, followed by 10 ml. of 0·1 M thioglycollic acid. Enough water was added to bring the solution to a final volume (including volume of enzyme solution) of 1 l. A purified phosphatase preparation from ox spleen (15 ml., specific activity about 700 units/mg. of protein nitrogen) was added and, after the addition of 25 ml. of toluene, the bottle was stoppered well and placed in an incubator at 37°. After 24 hr. a second portion (15 ml.) of enzyme solution along with thioglycollic acid (0·001 M final concentration) was added. A precipitate had begun to separate by this time. At the end of 48 hr., the precipitated protein was collected on the centrifuge and washed well with water.

For purification, the protein was suspended in water and brought into solution by the dropwise addition of dilute alkali (N) with continuous stirring. The solution was filtered through a layer of paper pulp on a Büchner funnel. The dephosphorylated casein was precipitated from this solution by the dropwise addition of N hydrochloric acid to pH 6·0. The precipitated protein was collected by centrifuging, washed repeatedly with water and dried with acetone. The air-dry product weighed about 15 g.

The protein is a white amorphous powder lighter than casein. It is relatively insoluble in water. It is, however, soluble in dilute alkali and its solubility in dilute acids is greater than that of casein. It is precipitated from its solution in alkali by acidifying to pH 6·0. There appears to be thus a shift in the isoelectric point of casein to the alkaline side during its dephosphorylation, presumably owing to the removal of the phosphoric acid groups.

The supernatant solution from dephosphorylated casein gave a small amount of precipitate (0·38 g.) when adjusted to pH 4·8. The filtrate from this precipitate gave, on half saturation with ammonium sulphate, additional amounts of a precipitate (0·4 g.). The nature of this product has not been investigated in detail.

During the dephosphorylation of casein, small amounts of acid-soluble nitrogen were also liberated, which corresponded to about 2% of the casein nitrogen. The nature of this product has been investigated by the paper-chromatographic technique.

Characterization of acid-soluble nitrogen formed during dephosphorylation of casein

A solution (50 ml.) containing 1 g. of casein, acetate buffer of pH 5·8 (0·1 M) and thioglycollic acid (0·001 M) was incubated with 1 ml. of a purified preparation of phosphoprotein phosphatase. After incubation at 37° for 24 hr., an additional amount (1 ml.) of enzyme was added. After 48 hr., the precipitated protein was collected by centrifuging. The precipitate was washed twice with water. The supernatant and the washings were combined and deproteinized by the addition of trichloroacetic acid. After being kept in a boiling-water bath for 15 min., the solution was filtered from the small amount of precipitate formed. The filtrate was shaken with several portions of ether in a separating funnel to remove trichloroacetic acid. The aqueous layer, after the final extraction, was drawn off and evaporated to dryness *in vacuo*. The small amount of residue obtained was dissolved in 2·5 ml. of water and filtered and the filtrate preserved for chromatographic analysis. Substrate blanks were carried out simultaneously. The experimental conditions were the same, with the difference that the substrate was incubated in the absence of the enzyme.

For paper-chromatographic examination, a 20–30 μl. portion of the test solution was applied as a spot on to a Whatman no. 1 paper (23 cm. × 40 cm.). The development of the chromatogram was carried out according to the ascending technique of Williams & Kirby (1948), with *n*-butanol–acetic acid–water (4:1:5) as solvent system. After the chromatogram had been run for 24 hr., the sheet was dried at room temperature. For the detection of the spots, the sheet was sprayed with a 0·1% (w/v) solution of ninhydrin in *n*-butanol. Spots were revealed by leaving the

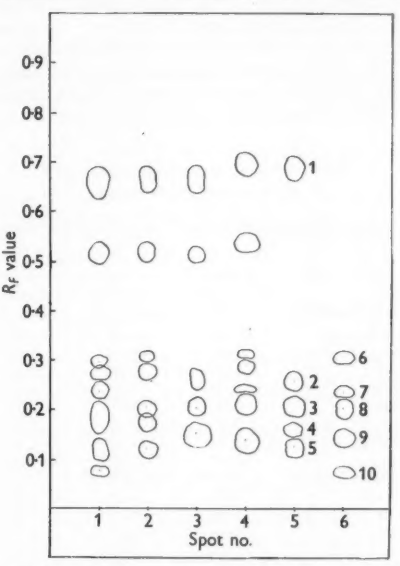

Fig. 1. Paper chromatogram of hydrolysates of peptides released during enzymic dephosphorylation of casein. Spots 1–4 represent hydrolysate of peptides having the R_F values indicated in the text. Spots 5 and 6 represent mixtures of authentic samples of the following amino acids: (1) isoleucine, (2) threonine, (3) serine, (4) arginine, (5) lysine, (6) alanine, (7) glycine, (8) glutamic acid, (9) histidine and (10) cystine.

paper in an oven at 100° for a few minutes. Four ninhydrin-positive spots were obtained in this way. With the solvent system used, they had the following R_F values: (1) 0·12, (2) 0·21, (3) 0·27 and (4) 0·89. Spot no. 4, which had the highest R_F value, was also found to be the most intense. In the blank experiment, a faint spot corresponding to an R_F of 0·89 was the only one obtained.

For further characterization, portions (about 20–30 μl.) of the test solution were applied as successive spots (five in number) to a Whatman no. 1 filter sheet (23 cm. × 40 cm.) and the chromatogram was run for a period of 24 hr., as described above. The sheet was taken out and the ninhydrin-positive spots, located with the aid of guide strips, were cut out from the paper. Spots having the same R_F values were combined and eluted from the paper with water. The eluates were hydrolysed in a sealed tube with 2N hydrochloric acid at 110° for a period of 24 hr. The product in each case was taken up in a small amount of water and analysed by paper chromatography, with the butanol–acetic acid–water solvent system. A diagrammatic representation of the chromatogram obtained with the acid hydrolysates of the different peptides is presented in Fig. 1. In the figure are also indicated the spots obtained with several authentic samples of amino acids.

Amino acid composition of dephosphorylated casein

Results of amino acid determinations on casein and dephosphorylated casein are given in Table 3. The values for dephosphorylated casein represent the average of results obtained with two different samples of the protein. The samples had more or less the same amino acid composition, the divergence in values being well within experimental error (below 2 % for chemical methods and below 5 % for the microbiological-assay methods). The analytical results presented in the table have all been corrected for moisture. The figures for serine and threonine include corrections made for the decomposition of these amino acids during acid hydrolysis of the protein, the correction factors worked out by Rees (1946) (100/89·5 for serine and 100/94·7 for threonine) having been applied for this purpose.

Table 3. *Amino acid composition of dephosphorylated casein*

Values are expressed in g./100 g. of the dry protein.

Constituent	Dephosphorylated casein	Casein	
		Present investigation	Literature value*
Total nitrogen	15·55	15·56	15·63
Total phosphorus	0·04	0·80	0·86
Alanine	3·26	3·38	3·00
Glycine	1·68	1·90	2·70
Valine	6·89	6·94	7·20
Leucine	9·43	9·54	9·20
Isoleucine	5·76	6·13	6·10
Proline	12·22	11·72	11·30
Phenylalanine	5·10	4·98	5·00
Tyrosine	7·21	6·32	6·30
Tryptophan	1·46	1·31	1·20
Serine	7·02	6·27	6·30
Threonine	5·27	5·28	4·90
Cystine	0·24	0·35	0·34
Methionine	2·99	2·93	2·80
Arginine	4·12	3·92	4·10
Histidine	3·06	3·03	3·10
Lysine	7·86	8·18	8·20
Aspartic acid	7·34	7·66	7·10
Glutamic acid	20·73	21·62	22·40

* Values taken from Gordon, Semmett, Cable & Morris (1949).

DISCUSSION

The enzymic dephosphorylation of casein is accompanied by the liberation of a small but significant amount of acid-soluble nitrogen. Paper-chromatographic examination of the nitrogenous product reveals that it is composed of a mixture of peptides (Fig. 1). Since the phosphatase preparation used in these studies was free from proteolytic activity and did not liberate acid-soluble nitrogen from egg albumin, it appears probable that the formation of acid-soluble nitrogen from casein is linked up with its dephosphorylation. Liberation of peptide material during enzymic dephosphorylation of casein has also been observed by Perlmann (1954*a*, *b*), and she has made the suggestion that, as with the disulphide bridge, phosphodiester and pyrophosphate linkages can cross-link peptides in the intact protein. Disruption of the linkage by means of a suitable phosphatase preparation would thus result in the liberation of peptides. The results obtained in the present investigation are in conformity with this suggestion. Additional evidence for this view is provided by the fact that the peptides formed during the dephosphorylation of casein contain, as common constituents, glutamic acid, isoleucine and serine—amino acids which are also present in the phosphopeptones obtained from casein by various workers (Rimington, 1941; Damodaran & Ramachandran, 1941).

The results presented in Table 3 show that the dephosphorylated casein has approximately the same amino acid composition as casein. Significant deviations are observed only with respect to a few amino acids. Thus the dephosphorylated protein contains somewhat higher amounts of serine, tyrosine and tryptophan and less cystine and glycine than casein. Dephosphorylated casein contains about 7 % of serine, as against the value of 6·3 % obtained for casein. These values include corrections made for the decomposition of serine during acid hydrolysis of the protein. The corrected values for casein may be low, in view of the greater lability to acid hydrolysis of serine combined as phosphoserine in phosphoproteins, as compared with that of free serine (Damodaran & Ramachandran, 1941; Nicolet, Shinn & Saidel, 1942; Mecham & Olcott, 1949). Thus Nicolet *et al.* (1942) give 7·38 % as the

probable serine content of casein, compared with 5·5% found on direct acid hydrolysis. The serine content of dephosphorylated casein is close to the value suggested by these workers for casein. These results taken with the findings of Mecham & Olcott (1949) indicate that the true serine content of phosphoproteins is best obtained when the estimation is carried out on the dephosphorylated protein.

Dephosphorylated casein prepared by the enzymic and chemical methods respectively differ in their content of most of the amino acids. Most striking perhaps are the observed differences in the contents of arginine, tryptophan, aspartic acid, cystine and the hydroxyamino acids and the presence of less than 1% of valine in alkali-treated casein (Macara & Plimmer, 1940). The alkali-dephosphorylated protein has also a lower nitrogen content. This has been attributed by various workers to the hydrolysis of the acid amide linkage (Rimington, 1927; Macara & Plimmer, 1940). The use of alkali as a dephosphorylating agent suffers from the additional disadvantage in that the casein molecule is split. Failure to obtain appreciable amounts of products, other than dephosphorylated casein, in enzymic digests of casein indicates that no such drastic changes take place during the enzymic dephosphorylation of casein. This view is also supported by results obtained on the amino acid composition of the enzymically dephosphorylated casein. The protein has also been found to be readily attacked by proteolytic enzymes (Sundararajan, 1956). Alkali-dephosphorylated casein is attacked by these enzymes with difficulty, possibly owing to some racemization having taken place during the treatment with alkali (Rimington, 1927). The results obtained in the present study thus indicate the advantages of enzymic over alkali dephosphorylation methods and emphasize the importance of phosphoprotein phosphatase as a specific tool for the study of dephosphorylated proteins.

SUMMARY

1. The preparation from ox spleen of a phosphoprotein phosphatase free from proteolytic activity and suitable as a dephosphorylating agent for casein is described.

2. The preparation and some properties of dephosphorylated casein are described. In the preparation of the protein, advantage has been taken of the insolubility of the dephosphorylated casein at pH 6·0, which is also the optimum pH for the enzymic dephosphorylation.

3. Acid-soluble nitrogen formed during the enzymic dephosphorylation of casein has been determined and the nature of the nitrogenous product investigated by paper chromatography.

4. Dephosphorylated casein has been analysed for amino acids. Comparison of the values with those obtained for casein shows that the amino acid composition of casein and of its dephosphorylated product are about the same.

5. It is concluded from these studies that during the enzymic dephosphorylation of casein the protein remains relatively intact and that the changes, if any, brought about by this treatment are not of a drastic nature.

6. The relative merits of alkali and enzymic dephosphorylation methods are discussed.

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Experiments on Electrical Migration of Peptides and Proteins inside Porous Membranes: Influences of Adsorption, Diffusion and Pore Dimensions

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This work was begun in the hope that separations depending chiefly on molecular weight could be achieved in the peptide series by methods of the kind used by Mould & Synge (1954*a*), who were able partially to resolve the homologous series of polymeric straight-chain dextrins under conditions of electrokinetic ultrafiltration in collodion membranes. The use of molecular-sieve effects in membranes for separations according to molecular weight is traditional for protein chemistry, in the techniques of simple dialysis and simple ultrafiltration. Signer, Hänni, Koestler, Rottenberg & von Tavel (1946) and others have developed apparatus, often rather complicated, for multiplying the enrichment effects observed in dialysis of low-molecular compounds so as to obtain actual separations. Craig & King (1955) have reported rates of passage through cellophan of amino acids, peptides, etc., and on the basis of these observations used a repetitive procedure, similar in design to fractional crystallization, to fractionate the partial hydrolysis products of tyrocidine according to their molecular weight. They showed that rates of dialysis are determined not only by diffusion rates and by molecular-sieve effects in the pores of the membrane but that other effects, probably due to electrical charges, must be invoked to explain their results. This point is further discussed below. Lathe & Ruthven (1956) have obtained useful fractionations of proteins, peptides, etc., according to molecular weight by chromatography on starch grains. Here, it seems, molecular-sieve effects cause different proportions of the water-filled spaces within the grains to be accessible to dissolved molecules of different sizes.

Mould & Synge (1954*a*) used the electroendosmotic flow of water inside, and parallel to the walls of, collodion membranes to effect chromatographic separations of dextrins. They concluded that these separations were primarily due to adsorption on the collodion surfaces, although molecular-sieve effects were also present. Exclusion by ultrafiltration seemed to set in rather abruptly for dextrins whose molecular weight exceeded a value which depended on the pore size of the membrane. As most proteins and peptides are positively charged and reasonably stable in weakly acid solutions, it was hoped that, under the conditions of Mould & Synge (1954*a*), they would migrate inside the membranes by electrophoresis and not simply with the electroendosmotic stream, and that the effects of chromatographic adsorption and molecular sieving, superimposed on the ionic migration, could then be studied by varying the porosity of the membranes. However, it was soon found that adsorption effects must be eliminated (see Results and Discussion) for satisfactory use of this type of apparatus.

In order to prevent adsorption, the collodion was denitrated. Solvent mixtures which would swell or dissolve collodion could then be used with the resulting cellulose membranes. The solvent mixture chiefly studied (phenol–acetic acid–water) was expected to displace peptides from the state of adsorption on cellulose. Such solvents tend also to break secondary-valence bonds between peptide molecules and thus act as dissociating agents (cf. Doty, Bradbury & Holtzer, 1956). I have for long doubted whether the substances generally used as protein precipitants or as solvents for extracting or dialysing amino acids and other non-protein material away from proteins can be relied on to extract from biological material peptides other than the simplest ones (Synge, 1953, 1955, 1956). It seems that, for purely chemical studies, solvents which dissociate secondary-valence bonds should be preferred, even

if they denature proteins and destroy biological activity, provided that they do not break primary-valence bonds in the molecules of the substances which are to be isolated and studied chemically. The use of such dissociating solvents thus has additional possible advantages for the extraction and subsequent purification of proteins and peptides.

Under the new conditions in the membranes, very gratifying zone separations dependent on molecular weight were obtained with a number of peptides and proteins. These were chosen because their chemical nature and molecular weights have been rather fully investigated. It has emerged that, on varying the porosity of the membrane, the relative migration rates of peptides and proteins are altered over a wide range of molecular weight. There was no sign of failure to enter the membrane, as with the dextrins in the experiments of Mould & Synge (1954*a*). Although further study is required before the new method can be used preparatively or for quantitative analysis, it seems worth publishing the rather crude observations so far made, so that others may make use of them. Smithies (1955) and Smithies & Poulik (1956) have observed somewhat similar effects on electrophoresis of proteins in stiff starch gels in the presence of borate and other buffers.

MATERIALS

L-*Arginine*. The monohydrochloride was used.

'*Salmine*' *sulphate*. A preparation of the protamine of *Oncorhynchus kisutch* obtained about 15 years ago from the Banting and Best Institute, Toronto, through Dr A. C. Chibnall, was used.

Insulin. Bovine insulin (Boots Pure Drug Co. Ltd., recrystallized, batch CBL 4245 LL), kindly given by Dr F. Sanger.

Gramicidin S hydrochloride. This was prepared from the same crude material as that described by Consden, Gordon, Martin & Synge (1947).

Tyrocidine hydrochloride. The Wallerstein preparation studied by Synge & Tiselius (1947) was used. This is a mixture of tyrocidines A, B, etc. (cf. Battersby & Craig, 1952*a*). In some preliminary experiments tyrothricin was used with similar results.

Cytochrome c. This was a preparation of equine origin (9E40918, E. R. Squibb and Sons, New York).

Ribonuclease. Pancreatic, crystalline ribonuclease (Armour) was used.

Lysozyme. This was prepared from egg white, crystalline (Armour).

Mercuripapain. The crystalline material (Kimmel & Smith, 1954; Smith, Kimmel & Brown, 1954) was used.

The last four preparations were kindly given by Professor Emil L. Smith.

METHODS

Membranes

Collodion membranes. These were prepared and characterized as described by Mould & Synge (1954*a*). Membrane A was prepared in the same way as membrane V there described. It showed similar rate of electroendosmosis and similar R_F value for a dextrin preparation of average degree of polymerization (DP) 33. It probably therefore had 'average pore radius' about 9 mμ. Membrane B was prepared in the same way as II, and showed R_F 0·7 and 0·12 for dextrin preparations of DP 33 and 160 respectively. It was 0·88 mm. thick and had average pore radius (rate of flow of water method) 18 mμ. Membrane C was prepared as for membrane V, but evaporation of the ethanol–ether was allowed to proceed for longer after the collodion had set before it was transferred to water. This gave a finer-pored membrane; dextrin of DP 33 had R_F 0·27, while dextrin of DP 160 was completely excluded. The membrane was 0·50 mm. thick and had average pore radius 6 mμ. It was not always easy to judge the stage of evaporation at which denser membranes such as C should be transferred to water and excessively impermeable membranes sometimes resulted. Perhaps the procedure of Adair (1956) would be more certain (see also Donnet & Roth, 1955).

Denitration of collodion membranes. The denitration procedure of Fuoss & Mead (1943) by the use of ammonium sulphide in excess of ammonia seriously weakened the membranes. On the kind advice of Mr F. D. Miles (cf. Miles, 1955), one of the procedures of Rassow & Dörr (1924), in which ammonium sulphide was used with excess of H_2S, was tried and proved satisfactory. Partial denitration in nitric acid (Alexander & Johnson, 1949) gave a membrane that softened somewhat in phenol–acetic acid–water and gave less good contrast on staining the zones (see below). A piece of collodion membrane (approx. 8 cm. × 20 cm.) was placed in the solution obtained by saturating with H_2S a mixture of ethanol (145 ml.), aqueous NH_3 (sp.gr. 0·880, 75 ml.) and water (to 250 ml.). After keeping for at least 3 hr. at room temp. the membrane was washed with changes of 50% (v/v) aqueous ethanol until no longer yellow, and then with 0·2 N aqueous acetic acid, in which it was stored. Collodion membrane A gave denitrated membrane AD, and so forth. Properties of the denitrated membranes are given in Table 1. Koz'mina & Starovoĭtova (1953) observed similar changes of membrane thickness and of pore radius on denitrating collodion membranes.

Migration experiments in membranes

These were done with the apparatus and procedure of Mould & Synge (1954*a*). The membranes were equilibrated with the solvent mixture to be used in the experiment for at least an hour before use, and the same solvent mixture was used in cathode and anode chambers. Equilibration of the

Table 1. *Properties of denitrated membranes stored in* 0·2 N *acetic acid*

Freshly blotted membranes were weighed and then dried *in vacuo* in a desiccator over H_2SO_4–soda lime at room temp. 'Average pore radius' was determined on wet membranes as by Mould & Synge (1954*a*), taking 1·53 g. cm.$^{-3}$ as the density of cellulose.

Membrane	Thickness (mm.)	Dry matter content (%)	'Average pore radius' (mμ.)
AD	0·75	24	6·5
BD	0·69	18	13·4
CD	0·41	32	4·7

liquid paraffin with the solvent was unnecessary. The peptide and protein solutions placed in the anode chamber were 1–2% (w/v). Development was continued with fresh solvent in the anode chamber for not less than 6 times the running-in time. The membrane strips were generally 1·3 cm. broad and about 7 cm. in effective length (anolyte to catholyte). Applied potential was 270 v. Currents were smaller with the solvents used here than with 0·2 N acetic acid. There was considerable browning of the phenolic solvent at the anode, but the resulting pigment showed no tendency to enter the membrane. All the compounds studied except arginine were detected, at the end of the run, by keeping the blotted membrane in 0·1% (w/v) aqueous flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid) with gentle agitation for 10 min. and then in several changes of water. When contrast was best, the strip was sketched or photographed and allowed to dry, although with some substances the strips could be stored wet without loss of staining contrast. Arginine was revealed by staining with 0·1% flavianic acid dissolved in 80% (v/v) aqueous ethanol, which solvent was also used for washing off the stain. Cytochrome *c* was visible at all times, and flavianic acid staining of its zones always coincided with the distribution of pink pigment, giving orange-coloured zones easily distinguished from those of other proteins. Moreover, on washing with water, cytochrome *c* appeared to lose flavianic acid more readily than did the other substances studied, giving zones coloured pink which persisted indefinitely on storage under water.

Filter-paper electrophoresis in phenol–acetic acid–water (1:1:1, *by wt.*)

This was done on Whatman no. 1 paper according to Kunkel & Tiselius (1951; cf. Ellfolk & Synge, 1955). Each spot was made with 2 μl. of a 5% (w/v) solution of the substance being studied. *N*-(2:4-Dinitrophenyl)ethanolamine (cf. Mould & Synge, 1954*b*) was used as a marker of electroendosmosis, siphoning, etc. No electroendosmosis was observed. For detection, the undried sheets were stained with ethanolic flavianic acid as above (not suitable for arginine, gramicidin S or tyrocidine), or the dried sheets were sprayed with ninhydrin in the usual way (and heated), or subjected to the Cl_2–starch–KI procedure of Rydon & Smith (1952). Cytochrome *c* was visible at all times. Where a substance being studied was detected by two or more methods, no discrepancies were noted in the positions of the spots.

Expression of mobilities

Ionic mobilities were throughout towards the cathode and are expressed in relation to the potential gradient measured in a straight line in the medium, assuming the ions also to be moving in a straight line. No correction for electroendosmosis was necessary. Migrations were usually measured from the anode end of the membrane to the sharp edge of the zone.

RESULTS

Conditions for electrical migration of peptides in a membrane

All these experiments were done with collodion membrane A or with the derived denitrated membrane AD.

Collodion membrane in saturated urea solution. In 0·2 N aqueous acetic acid 'salmine' migrated at 10×10^{-5} cm.^{2} v^{-1} sec.$^{-1}$. In the same solvent saturated with urea it had the same mobility, but the zone (which had a sharp leading edge in the absence of urea, perhaps due to adsorption), had a sharp tailing edge, suggestive of pH and conductivity effects (cf. Svensson, Benjaminsson & Brattsten, 1949). Gramicidin S in 0·2 N acetic acid saturated with urea entered only the first millimetre or so of membrane, which became shrunken and translucent. During the experiment (17 min.) the current fell from 2 to 1 mA. Tyrothricin gave the same result, except that shrinkage and fall of current were more marked.

Denitrated membrane in saturated urea solution. In 0·2 N aqueous acetic acid sat. with urea, 'salmine' had mobility $4{\cdot}3 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$, showing a sharp trailing edge, whereas gramicidin S had mobility $1{\cdot}2 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$, the zone showing a sharp leading edge and extending diffusely back towards the origin. Tyrothricin did not enter far, causing shrinkage of the 2 mm. of membrane nearest the anode, with fall of current from 1·1 to 0·6 mA.

Denitrated membrane in phenol–acetic acid–water (1:1:1, *by wt.*). 'Salmine', gramicidin S and tyrocidine gave fairly compact zones with the trailing edges the sharper, having respective mobilities 2·7, 1·9 and $0{\cdot}9 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$. These are in similar proportion to the relative mobilities determined in the same solvent on filter paper (Table 2). Current was constant (0·3 mA approx.) in all three experiments. A mixture of the three substances under the same conditions gave three well-resolved zones. These observations were taken to indicate that adsorption had been eliminated and that, with the peptides in question, membrane AD did not show much selective retardation dependent on diffusion or molecular-sieving effects.

Effect of porosity of membrane on migration of peptides and proteins

In these experiments, the relative ionic mobilities of several substances of fairly well-established mol.wt. were compared in phenol–acetic acid–water (1:1:1, by wt.) on filter paper, in the coarser membrane BD and in the finer membrane CD. Each substance was compared in mobility with cytochrome *c* (by running a mixture of the two in the membrane experiments, and by having spots side by side on filter paper). By observing the membranes before and after staining with flavianic acid it was easy to distinguish by colour the cytochrome zone from that of the substance being tested, and greater reliance can thus be placed on the relative mobilities than if they had been determined in separate experiments, where fortuitous variations of applied

voltage, membrane porosity, etc., might have misled. As mentioned under Methods, cytochrome *c* showed no sign of dissociation into pigment and protein moieties, as occurs with haemoglobin or myoglobin in acidic organic solvents. This can be attributed to the primary-valence bonding between pigment and protein (Theorell, 1938; Tuppy & Bodo, 1954). The results are set out in Table 2. It is seen that, while in membrane BD there is some indication of influence of molecular weight on mobility, in the finer-pored membrane CD this is very much greater.

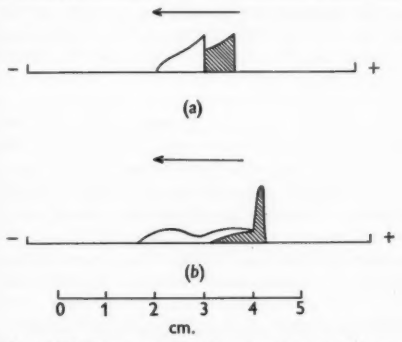

Fig. 1. (*a*) Displacement effect in migration of a mixture of gramicidin S and cytochrome *c* in membrane BD. Run, 53 min. (*b*) Interaction of tyrocidine and cytochrome *c* during migration of a mixture in membrane CD. Run, 210 min. Hatched areas indicate distribution of visible pigment, unhatched of material staining with flavianic acid only, as assessed by eye.

Where, in these experiments, two zones lay close together, displacement effects of the kind observed by Svensson *et al.* (1949) were noticed. With increasing concentration of migrating substance, there are local changes in pH and conductivity (which determines potential gradient), which tend to decrease the migration rate. Zones therefore have sharp trailing edges and diffuse leading edges. Fig. 1 (*a*) shows diagrammatically the displacement effect of gramicidin S on cytochrome *c* in membrane BD. The change of conditions at the trailing edge of the gramicidin S zone 'displaces' what would otherwise be the diffuse leading edge of the cytochrome zone. Fig. 1 (*b*) illustrates the type of interaction between cytochrome *c* and tyrocidine or insulin, which was observed in membrane CD. Approximate migration rates of the intermediate zones are given in a footnote in Table 2.

DISCUSSION

Adsorption effects in membranes

In the experiments aimed at establishing conditions for the ionic migration of peptides in collodion membranes, the pore size used (membrane A) permitted passage of dextrins up to at least mol. wt. 5000 and should therefore have been ample for the passage of gramicidin S and tyrocidine, if not for salmine. In fact, salmine was found to migrate freely in the membrane in dilute acetic acid whether or not urea was present, while gramicidin S and tyrocidine did not migrate in the same solution saturated with urea (in order to dissolve them). With

Table 2. *Relative mobilities of substances in filter paper and in membranes of different porosity, with phenol–acetic acid–water* (1:1:1, *by wt.*) *as solvent*

A, mobility relative to that of cytochrome *c* in same membrane. *B*, mobility relative to that of substance itself in filter paper. Cytochrome *c* had average mobility: 2·9 (range 2·8–3·0) $\times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ in filter paper; 1·6 (range 1·3–1·9) $\times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ in membrane BD; 0·47 (range 0·28–0·82) $\times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ in membrane CD.

		Filter paper		Membrane BD		Membrane CD	
	Mol.wt.	*A*	*B*	*A*	*B*	*A*	*B*
L-Arginine*	174‡	1·27	1	1·57	0·68	2·60	0·33
Gramicidin S	1 142‡ (1)	0·86	1	1·20	0·77	2·57	0·48
Tyrocidine	1 270‡ §(2)	0·58	1	0·72	0·68	1·75†	0·49
Insulin	5 734 (3)	0·89	1	0·94	0·58	1·79†	0·33
Ribonuclease	14 000 (4)	0·92	1	1·00	0·60	0·87	0·15
Cytochrome *c*	13 000–16 000 (5)	1	1	1	0·55	1	0·16
Lysozyme	14 000–17 000 (6)	0·96	1	0·96	0·55	0·94	0·16
Mercuripapain	>21 000 (7)	0·91	1	0·85	0·52	0·61	0·11
'Salmine'	3 800–6 800 (8)	1·32	1	—	—	—	—

* This gave very spreading zones. Figures refer to trailing edge only.

† These figures refer to the fastest, cytochrome-free zone. There was also an intermediate, mixed zone, having $A = 1{\cdot}29$ for tyrocidine and $A = 1{\cdot}21$ for insulin. See Text.

‡ Free base. § Tyrocidine A.

(1) Battersby & Craig, 1951; (2) Battersby & Craig, 1952*b*; (3) Ryle, Sanger, Smith & Kitai, 1955; Sluyterman, 1955; (4) Hirs, Stein & Moore, 1954; Edsall, 1953; (5) Wyman, 1948; Edsall, 1953; (6) Fevold, 1951; Edsall, 1953; (7) Smith, Kimmel & Brown, 1954; Smith, Stockell & Kimmel, 1954; (8) Phillips, 1955.

these last peptides, the current fell and the membrane shrank seriously at the anode end. An obvious explanation could be that gramicidin S and tyrocidine, but not salmine, are adsorbed on the collodion surfaces, conferring a positive charge and reversing the direction of electroendosmosis in that portion of the strip of membrane. Water continues to move towards the cathode from ahead of the adsorbed zone but back towards the anode inside the zone, so that shrinkage of the membrane, with drying out and total blockage, occurs. The next step was thus to try to eliminate adsorption. Denitration of the membrane was found to permit passage of gramicidin S in dilute acetic acid saturated with urea (adsorption on cellulose being presumably less than on cellulose nitrate), but tyrocidine still did not migrate and caused shrinkage of the membrane. As the membrane had been converted into cellulose, organic solvents could now be used in it without swelling or weakening it, and a mixture of equal parts of phenol, acetic acid and water was found to permit migration of tyrocidine without shrinkage of the membrane.

Fridrikhsberg & Gutman (1953; cf. Toman, 1954; Fridrikhsberg & Gutman, 1954) have stressed the idea, apparently not previously enunciated, that, where ionic material in solution is migrating in a coarsely porous membrane and is also strongly adsorbed on the pore walls, it will confer a charge on the walls such as to promote electroendosmotic flow of liquid relative to the membrane exactly equal and opposite to the migration velocity of the dissolved ions relative to the liquid. They showed that various dyes could not migrate across collodion diaphragms under conditions of free electroendosmosis, although they migrated readily when electroendosmosis was prevented by hermetically sealing the two chambers of their diaphragm cell. They observed similar effects with serum proteins. Thus, under conditions where electroendosmosis can occur freely, as with the apparatus used in the present experiments, it is useless to expect ionic transport of substances which are appreciably adsorbed on the membrane. The same applies to most arrangements using electrical transport in diaphragm cells, and an example can usefully be given from work done in this Laboratory. Synge (1951) described a fractionation of grass juice by electrical transport in a diaphragm cell. Most of the 'bound' amino acids present did not migrate towards the anode through a membrane of formolized parchment at pH 6, although glutamic acid, aspartic acid and various plant acids migrated readily. As the bound amino acids also did not migrate towards the cathode through cellophan at pH 3, they were judged to be devoid of ionizing groups. More recent experiments by Mr J. C. Wood and me (unpublished work) have shown that much of this 'bound amino acid fraction' is actually acidic in nature and migrates freely towards the anode on electrophoresis in silica jelly at pH 6. Presumably adsorption of these compounds on the parchment membrane produced electroendosmosis sufficient to prevent their own migration, while not preventing that of anions of greater mobility. Although the effect in the present instance was deleterious, there seem to be possibilities, based on the ideas of Fridrikhsberg & Gutman, for making membranes for diaphragm cells that will not simply be selective for cations or anions as such but will permit selective passage of ions exceeding a definite threshold of mobility, which can be controlled by the chemical nature of the membrane or of substances adsorbed thereon.

Adsorption effects of this kind should not seriously hinder passage of materials by diffusion or mechanical ultrafiltration through relatively coarse membranes, but in fine-pored membranes they can be expected to hinder the passage of the compound in question because the concentration of dissolved ions will be greatly diminished in the region of the electrical double layer which occupies an increasing proportion of the volume of the pores. The observations of Craig & King (1955) on relative rates of diffusion of differently charged amino acids and peptides through cellophan bear out the occurrence of such effects, and it is interesting to note how slowly tyrocidine passed through cellophan, even in 50 % (v/v) aqueous acetic acid, in view of the strong adsorption of tyrocidine suggested by the present experiments (see also Grandjean, 1952).

Other factors influencing migration in porous media

The experiments on the influence of membrane porosity on migration rates were all done with phenol–acetic acid–water, in view of the good results with tyrocidine and because the mixture is an excellent solvent for all the substances studied so far, is volatile and free from nitrogen (which will be useful in analytical and preparative work) and is unlikely to attack primary-valence bonds in the compounds being studied. Doubtless, future work will reveal other useful solvent media. With filter paper, there was negligible electroendosmosis in phenol–acetic acid–water [with *N*-(2:4-dinitrophenyl)ethanolamine as marker], nor was there noticeable electroendosmotic movement of solvent in the experiments with denitrated collodion membranes. The ionic migration on filter paper of the compounds studied showed a surprisingly slight range of mobilities in view of the great range of the ratio of number of ionizing basic groups in the molecule to molecular weight (cf. Table 2). Perhaps, in such solvents, dielectric effects decrease greatly the extent of ionization of basic groups when they are close together in the molecule, or there may be

rather tight binding of oppositely charged (acetate) ions.

It is clear that the effects on migration rates due to the use of a more finely pored membrane (Table 2) are closely correlated with molecular weight. It is not as yet clear what is the mechanism of this effect. Westhaver (1947; cf. Synge, 1949) discussed formally the migration of ions in a packed column against counterflowing liquid, in relation to the separation of potassium isotopes, and used a 'theoretical plate' treatment to take account of diffusion and convection in the flowing liquid as factors broadening the zones. However, he did not pay attention to the possible influence of the packing on the absolute or relative migration rates of the zones of solutes undergoing separation. Kunkel & Tiselius (1951) have treated the effect of the packing as increasing the path length that must be travelled by the migrating ion while at the same time decreasing the potential gradient, which is less when measured along this path than when measured in a straight line along the column. This idea has been attacked by McDonald (1955; cf. McDonald, Lappe, Marbach, Spitzer & Urbin, 1953), who proposes a 'barrier' theory, in which the electrical field in the liquid is considered to be uniform in strength and direction throughout; migrating ions encounter mechanical obstructions to their progress, however, at more or less frequent intervals. McDonald criticizes Kunkel & Tiselius on the practical ground that their theory does not predict differences in the relative retardation of molecules of different molecular weight. He gives (p. 60) a diagram of molecules of different sizes hitting paper fibres with different frequencies, which is so grossly distorted in relative scale as to be misleading; he also gives examples of differential retardation of different substances by different types of filter paper, ignoring the points: (i) that small molecules can penetrate cellulose fibres while large ones cannot; (ii) that electrical transport of small molecules within cellulose fibres will be much influenced by the electrochemical nature of the small molecule, as is well known from experiments on transport of ions across cellophan membranes. McDonald further criticizes Kunkel & Tiselius on theoretical grounds which may be valid if the packing of the column itself has appreciable conductivity, but are certainly not valid if the packing is an insulator. McDonald complicates his arguments by using the expression 'thermodynamic activity' in a sense quite different from that in general use.

The essential difference between these two approaches is whether the field is regarded as curving round the packing (an insulator) or passing straight through it (in which case it must be penetrable by the majority of migrating ions). There will be limiting cases where one or the other approach is more strictly applicable. For the practical objective of relating mobilities in packed media to those in free solution, conductivity measurements have been used by both groups as the basis of conversion, with essentially similar results.

A reasonable explanation for the effects seen in the present experiments could be that during ionic migration the larger molecules are obstructed on encountering barriers of regenerated cellulose through which hydrogen and acetate ions, carrying the greater part of the current, can migrate freely. The larger molecules would then escape from these situations by circuitous routes, at rates depending on their diffusion coefficients. Information on the detailed structures of collodion membranes has been given by Bugher (1953), Beutelspacher (1954), Helmcke (1954), Maier & Beutelspacher (1954*a*, *b*) and Spandau & Zapp (1954). When the pore size approaches molecular dimensions still more closely, the 'barrier' effect of McDonald or 'frictional' effects of Tiselius & Flodin (1953) must come into operation, and are probably tautologous with the expression 'molecular-sieve' effects. Even here, diffusion presumably plays a part in extricating molecules from situations in which they have become immobilized. This is implicit if one extends to ionophoretic migration the treatment by Mackie & Meares (1955) of the diffusion of ions inside ion-exchange resins.

Applicability of the new procedure

Detailed analysis of the mechanism of electrical transport in porous media must clearly await further experimental and theoretical studies. The present work shows, however, that, regardless of mechanism, resolutions dependent mainly on molecular weight or diffusion behaviour can be realized with peptides and proteins in such media. Reasonably well-characterized chemical entities were selected for the present study, and it was pleasing that they arranged themselves mostly in the sequence to be expected from what is known of their molecular weights. All the substances studied, moreover, migrated as reasonably compact, single zones (although, in view of the slight range of mobilities in phenol–acetic acid–water, migration as a single zone is not a very serious test of homogeneity). With the mixtures (cytochrome *c* + insulin) and (cytochrome *c* + tyrocidine) the formation of mixed zones of intermediate mobility (cf. Grassmann, 1953; Grassmann & Hübner, 1953; Synge, 1956) suggests reversible complex formation, perhaps with dissociation of the cytochrome into submolecules which combine with tyrocidine or insulin; the resultant complexes would migrate faster than the cytochrome in membrane CD but at a similar rate to it in membrane BD. This phenomenon seems worthy of more detailed study, since

both tyrocidine and insulin have loci in their molecules at which aromatic amino acids are grouped closely together. These loci might enter into combination with similar loci in cytochrome sub-molecules which normally combine with one another. Diffusion experiments in free solution should yield relevant results. Tint & Reiss (1951) reported the formation of a complex between cytochrome *c* and the peptide antibiotic bacillomycin *b*, and studied its electrophoretic behaviour.

The new procedure has obvious possibilities for fractionating according to molecular weight polymeric electrolytes which, in free solution, migrate with ionic mobility independent of molecular weight. It should also be useful in analysing whole tissues for polypeptides (cf. Synge, 1953, 1955, 1956). Compounds migrating faster than cytochrome *c* in membranes such as CD can be expected to be substantially more basic in character, or of lower molecular weight, or both. It might be possible to increase the scale of working by conducting the migrations with the field perpendicular to membranes packed tightly together in a pile. Improved arrangements are also needed for preventing electrode reactions from attacking solvents and materials undergoing analysis.

SUMMARY

1. In order to obtain ionic migration of peptides and proteins inside porous membranes, adsorption of the migrating substance on the structure of the membrane must be eliminated.

2. Favourable conditions for migration resulted from using a phenol–acetic acid–water mixture as solvent inside membranes of denitrated collodion.

3. In free solution in the same solvent a number of polypeptides and simpler proteins had unexpectedly similar cationic mobilities.

4. Experiments on ionic migration in finely pored membranes showed a strong selective-retardation effect; those peptides and proteins of higher molecular weight were the more strongly retarded. Possible mechanisms for this effect are discussed.

5. Evidence was found for complex formation by cytochrome *c* with insulin and with tyrocidine.

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Improved Method for the Preparation of Crystalline β-Lactoglobulin and α-Lactalbumin from Cow's Milk

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Crystalline β-lactoglobulin is usually prepared from cow's milk by modifications of the method of Palmer (1934), based on exhaustive dialysis of the 'lactalbumin' fraction isolated by various salting-out procedures. A full description of this type of preparation has recently been given by Larson & Jenness (1955), who list earlier references. The 'lactalbumin' fraction contains another major whey protein, α-lactalbumin, which can be isolated from the β-lactoglobulin mother liquor (Gordon & Semmett, 1953; Gordon, Semmett & Ziegler, 1954; Gordon & Ziegler, 1955).

It has been noted (Gordon & Ziegler, 1955) that 'incomplete removal of β-lactoglobulin appears to hinder the subsequent crystallization of α-lactalbumin', and we have found that the presence of α-lactalbumin tends to interfere with the formation of β-lactoglobulin crystals, and to affect their purity adversely. Furthermore, the lengthy process of crystallizing the β-lactoglobulin is time-wasting when preparation of the α-lactalbumin is the sole objective. A clean separation of the two proteins at an early stage in their preparation would thus provide obvious advantages. Zweig & Block (1954) attempted segregation of the proteins after precipitation with ferric chloride, but achieved only partial separation and a much lower yield of α-lactalbumin than that obtainable by the improved procedure of Gordon *et al.* (1954).

The present paper, which forms part of a general study of whey proteins, describes a simple method for the early and practically complete separation of β-lactoglobulin from α-lactalbumin, and for the crystallization of the separated proteins by steps which can be carried out simultaneously or independently with a considerable saving in time. The milks of individual cows are now known to contain either a mixture ($\beta_{1,2}$) of two different β-lactoglobulins, or only one (β_1) or the other (β_2) of these (Aschaffenburg & Drewry, 1955). The method is equally applicable to such milks and to their mixture in bulk milk, except for minor modifications during the final stages of the preparation of crystalline β_2-lactoglobulin.

EXPERIMENTAL

Milk. All samples were of fresh, whole milk from cows of the Institute herds. Fractionation of 2–10 l. of the uncooled milk was started within a few hours of milking.

Reagents. All chemicals used in the fractionation procedures were of analytical reagent quality. This avoids loss of time through re-working, which was found to be necessary when less pure reagents were employed. Distilled water was used in all operations, as the presence of heavy metal ions in tap water led to the formation of discoloured protein precipitates.

Dialysis. This was done in narrow bags made from seamless Visking cellulose tubing by tying one end into a double knot, filling the bag, and securing the other end in the same manner. By leaving no air-space, undesirable increases in volume were reduced to a minimum. Although pressure inside the bags may increase considerably, no bursts have occurred with narrow tubing (not exceeding ¾ in. inflated diameter). If necessary, more than one narrow bag was filled rather than one of larger diameter. The bags were made to rotate during dialysis.

pH. This was determined with a glass electrode and a Pye Universal pH meter. All values refer to a temperature of 20°.

Nitrogen. Determinations were made by the semi-micro Kjeldahl method of Rowland (1938), with a selenium catalyst. Heating was continued for 1 hr. after the digests had cleared.

Working temperature. Unless specifically stated, all operations were done at room temperature (approx. 18°). In our experience, this does not affect the preparations adversely, and has, in fact, distinct advantages during the crystallization of β-lactoglobulin which, in confirmation of Palmer (1934), was found to be easier and quicker at room temperature than at that of the cold room.

Paper electrophoresis. This convenient method was used widely during the development stages to test the efficacy of the various fractionation steps. At each step, the proteins remaining in solution were salted out, and concentrated solutions of these as well as of the protein precipitated at the particular stage were analysed to obtain a complementary picture. All concentrates were prepared for electrophoresis by dialysis in seamless Visking cellulose tubing of $\frac{8}{32}$ in. inflated diameter against running tap water. Prolonged dialysis was unnecessary, a minimum time of 90 min. being sufficient to obtain satisfactory electrophoretic resolution. All runs were made in an apparatus of the type described by Flynn & de Mayo (1951), applying 230 v for 16 hr. at room temperature, on Whatman no. 3 MM paper at pH 8·6 in barbitone buffer, *I* 0·05. The test samples were applied on the cathodic side, 7·5 cm. from the apex. Bromophenol blue was used for staining, and electrophoretic diagrams were obtained by direct scanning of the untreated strips. As will be discussed elsewhere, these diagrams are not suitable for quantitative evaluation of the concentration and distribution of whey proteins.

METHODS OF FRACTIONATION

Isolation of the 'lactalbumins'

Whole milk is warmed to about 40°, and anhydrous Na_2SO_4 (20 g./100 ml.) added with stirring. After the salt has dissolved, and the temperature has fallen below 25°, the mixture is filtered through fluted Whatman no. 4 papers. There is no need to prepare separated milk, since the presence of the milk fat improves the rate of filtration. The filtrate (F 1) contains the 'lactalbumins', as shown in Fig. 1*a*. The precipitate containing the globulins and proteose–peptone components of milk, in addition to the casein and fat, is discarded. If the globulins and proteose–peptone components are required, the casein and fat may first be separated by acid precipitation, and the other components precipitated as above after the pH has been adjusted to 6·8.

Separation of β-lactoglobulin and α-lactalbumin

The volume of filtrate F 1 is measured, and conc. HCl (1 ml./100 ml.) added with vigorous stirring. This reduces the pH to approximately 2, causing rapid precipitation of all the protein constituents with the exception of the β-lactoglobulin, as shown in Fig. 1*b*, *c*. If the preparation is left to stand at this stage, Na_2SO_4 tends to crystallize out, and should be redissolved by gentle warming, as a reduction of its concentration affects the subsequent salting-out of the β-lactoglobulin fraction. The precipitate (P 2) is best separated from the supernatant (F 2) by prolonged centrifuging, but the precipitate does not always pack well. In that case, it must be obtained by filtration through a fluted Whatman no. 4 paper. The separated proteins can now be worked up simultaneously. Their subsequent behaviour suggests that they are unaffected by the

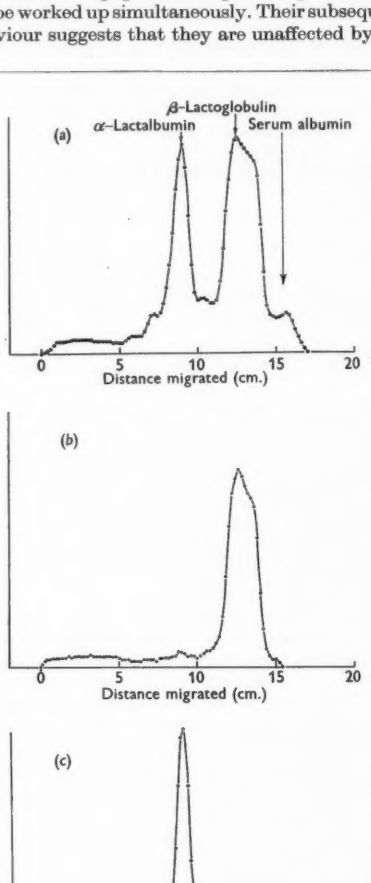

Fig. 1. Electrophoretic pattern of (*a*) the 'lactalbumins', and of (*b*) the β-lactoglobulin in the supernatant, and (*c*) the α-lactalbumin in the precipitate obtained after acidification to pH 2.

treatment at pH 2, a finding in line with the work of Zweig & Block (1954) whose procedure involves a lengthy dialysis under even more acid conditions.

Crystallization of β-lactoglobulin

The volume of supernatant F 2 is measured, and the pH brought to about 6 by dropping conc. NH_3 soln. (about 0·6 ml./100 ml.) into the well-stirred solution. The β-lactoglobulin is then precipitated with $(NH_4)_2SO_4$ (20 g./100 ml.). After the protein has flocculated, it is filtered by suction through a thin pad of Hyflo Super-Cel deposited on a Whatman no. 1 filter paper. The filtrate is discarded, and the filter cake triturated with a little water to produce a paste just sufficiently fluid to permit transfer to a dialysing bag. It is desirable to reduce the volume of supernatant F 2 to about one-eightieth to one-hundredth at this stage. The bag is closed and left stationary in water saturated with toluene. After dialysis overnight, the contents of the bag are filtered by suction through Whatman no. 1 paper to remove the filter aid. The pH of the clear filtrate is adjusted to 5·8, toluene added, and dialysis continued for 24 hr. by rotating the bag in several changes of water. Without rotation, the period of dialysis should be increased to at least 48 hr.

The pH is now reduced to 5·2 with dil. HCl, which causes the solution to turn cloudy. Preparations of β_2-lactoglobulin may remain almost clear at this stage, and their treatment will be discussed separately. With all other forms of β-lactoglobulin, the protein separates on further dialysis within a few hours in the form of orthorhombic plates or of an oil that can be converted quickly into crystals by seeding. Crystals from bulk milk preparations, and crystals of β_1- or $\beta_{1,2}$-lactoglobulin may be used indiscriminately for seeding, as they are isomorphous (Green, North & Aschaffenburg, 1956). Without seeding, crystallization may be delayed, although it has always occurred within 24 hr. Once crystals have formed, most of the protein separates within 24 hr. when, with concentrated solutions, as little as 5–10 % of the nitrogenous matter has been found to remain in solution.

According to Larson & Jenness (1955) the first crystalline product obtained by the conventional method should be recrystallized at least four times. This is not necessary with crystals prepared by the present method, since the starting material (F 2) is virtually free from proteins other than β-lactoglobulin (cf. Fig. 1*b*). With preparations made from bulk milk, the yield of crystals (dry wt.) has been 1·3–1·5 g./l. of filtrate F 1, i.e. of the same order as that reported by Palmer (1934) of 1·5 g./l. of whey. Similar yields have been obtained in preparing $\beta_{1,2}$- and β_1-lactoglobulin.

Crystallization of β_2-lactoglobulin. As mentioned above, solutions of β_2-lactoglobulin tend to remain almost clear when the pH is adjusted to 5·2. On further dialysis, they are slow in forming an oil and in crystallizing unaided, and the yield of crystals is usually disappointing. Two factors combine to cause this anomalous behaviour. The concentration of β_2-lactoglobulin in the milk of cows producing this type tends to be rather low (Aschaffenburg & Drewry, in preparation), and it is, therefore, particularly important to prepare as concentrated a solution as possible of the salted-out protein, and the diamond-shaped crystals formed by spontaneous crystallization have a relatively high solubility. Fortunately, β_2-lactoglobulin occurs in two forms, the diamond-shaped crystals which are monoclinic, and orthorhombic crystals which are less soluble than the former (Green *et al.* 1956). To obtain β_2-lactoglobulin in better yield, it is therefore advisable to proceed in the usual manner and to seed the solutions with orthorhombic crystals. If monoclinic crystals are required, they can be obtained with ease by dialysis against water of highly concentrated solutions of the crystals in dil. NaCl.

Crystallization of α-lactalbumin

As shown in Fig. 1*c*, the precipitate (P 2) obtained at pH 2·0 contains the α-lactalbumin together with the blood-serum albumin normally present in milk (Polis, Shmukler & Custer, 1950), two minor whey-protein constituents, and some residual β-lactoglobulin. The precipitate is dissolved in water with the help of dil. NH_3 soln. to one-tenth of the volume of filtrate F 1. Any residual β-lactoglobulin is now removed by reducing the pH to 3·5 with dil. HCl, when this protein remains in solution. The centrifuged precipitate is dissolved in one-quarter of the previous volume of dil. NH_3 soln., and dil. HCl added to pH 4·0. At this pH, the α-lactalbumin precipitates slowly, and the sample is best stored in the cold overnight. After warming to room temperature, the precipitate is spun down in the centrifuge. To the separated supernatant, solid $(NH_4)_2SO_4$ (11·5 g./100 ml.) is added to bring down additional α-lactalbumin. The supernatant from this precipitate contains the blood-serum albumin, and appears to be a more convenient starting material for the isolation of this protein than the mother liquor obtained after crystallization of β-lactoglobulin by the conventional method which was used by Polis *et al.* (1950).

The α-lactalbumin precipitates are again dissolved in dil. NH_3 soln., and made up approximately to the previous volume. The pH is adjusted to 6·6, and an equal volume of saturated $(NH_4)_2SO_4$ soln., previously brought to the same pH with conc. NH_3 soln., is added under stirring, as recommended by Gordon & Semmett (1953). After removal of the discoloured precipitate by filtration through

Whatman no. 4 paper, the α-lactalbumin is crystallized by their method of further slow addition of saturated $(NH_4)_2SO_4$ soln., adding about one-and-a-half times the volume required for half-saturation.

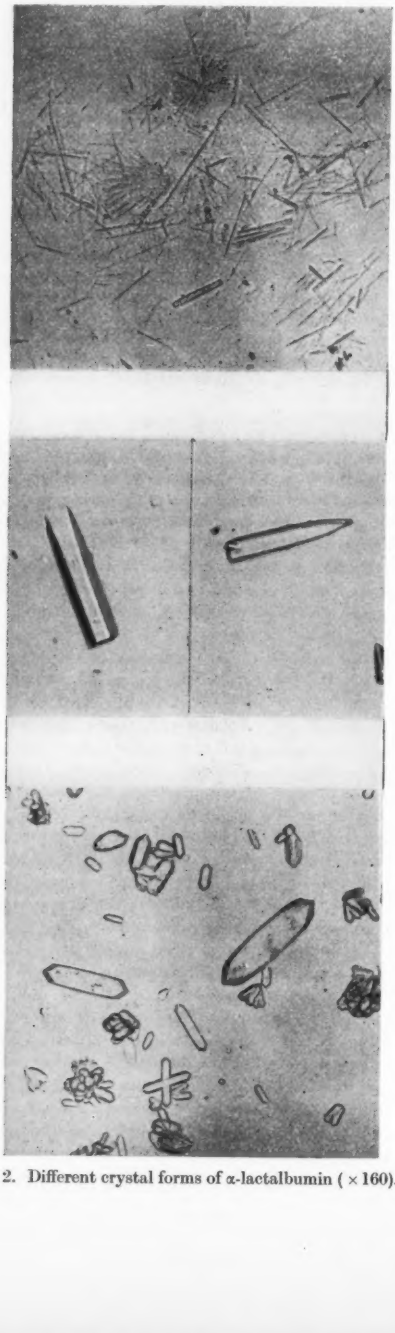

Fig. 2. Different crystal forms of α-lactalbumin (×160).

The first crystalline product is usually far from pure, the two minor protein constituents shown in Fig. 1*c* forming the main contaminants. The faster-moving of these does, in our experience, cling tenaciously to α-lactalbumin, and is difficult to remove even by several paired reprecipitations at pH 4·0 and recrystallizations at pH 6·6 in the manner recommended by Gordon *et al.* (1954). Attempts to remove this contaminant by other fractionation procedures have been unsuccessful. Gordon & Ziegler (1955) have reported a yield of once-recrystallized α-lactalbumin (anhydrous, salt-free basis) of 0·36 g./l. of whey. Our yields (dry wt.) at this stage have varied from 0·38 to 0·43 g./l. of filtrate F 1.

Large, well-defined crystals are difficult to obtain by the method described above. They can be prepared with ease by adding saturated $(NH_4)_2SO_4$ soln. to the half-saturated filtrate until a faint cloudiness develops, by transferring the solution to a crystallizing dish, and by allowing the protein to crystallize slowly as the $(NH_4)_2SO_4$ concentration increases through evaporation. When crystallization is complete, as indicated by the absence of turbidity after addition of saturated $(NH_4)_2SO_4$ soln. to a filtered sample, the crystals are taken up in 0·7 saturated $(NH_4)_2SO_4$ soln. to dissolve any salt crystals formed during evaporation. Application of this technique has produced three types of α-lactalbumin crystals (Fig. 2). They formed in a haphazard manner when allowed to crystallize spontaneously, and seeding with one type led more often than not to the formation of one of the other types in the evaporating solutions. A photomicrograph of bullet-shaped crystals is to be found in the paper by Zweig & Block (1954).

For a protein, α-lactalbumin has a relatively low molecular weight of about 16 000 (Gordon & Semmett, 1953). This may account for the finding that on ultrafiltration, though not materially on dialysis, the dissolved protein passes through Visking tubing with some ease. We have, in fact, found it possible to prepare crystalline α-lactalbumin from a straight ultrafiltrate of milk.

SUMMARY

1. An improved method for the crystallization of the β-lactoglobulins and α-lactalbumin of cow's milk is described in full. Its main advantage lies in the initial separation of the two types of protein by a simple acidification procedure, thereby permitting simultaneous or independent working-up of the separated fractions.

2. The method is equally applicable to bulk milk, and to the milk of individual cows containing β_1- or β_2-lactoglobulin either singly or in combination.

3. Yields of β_2-lactoglobulin may be improved by crystallizing it in the orthorhombic rather than the monoclinic form.

We are grateful to Mr N. Gruber for the photomicrographs.

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Connective Tissue Growth Stimulated by Carrageenin

1. THE FORMATION AND REMOVAL OF COLLAGEN

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Although connective tissue growth has been studied intensively by histological methods (for review see Cameron, 1952) and to a lesser degree by electron-optical methods, very few biochemical studies are available in the literature. Kodicek & Loewi (1955) have studied the granulation tissue in regenerating tendon, and Dunphy & Udupa (1955) have reported on a combined biochemical and histological study of connective tissue formation in healing wounds. In neither of these studies was any attempt made to fractionate the collagen into the three types known to exist in developing connective tissue. The paucity of information is probably due to the fact that the amount of new tissue formed in a given period in normal circumstances is small and cannot be separated from pre-existing tissue. Where the new tissue is separable, as for example in healing wounds, the amount of tissue formed is insufficient for detailed biochemical studies. Recently, however, Robertson & Schwartz (1953) have reported a method of producing comparatively large amounts of easily separated connective tissue. This method consisted of injecting a solution of carrageenin subcutaneously into the lower abdominal regions of guinea pigs. Carrageenin is a sulphated polygalactose which is extracted from the seaweed Irish moss (*Chondrus crispus*). Here again, however, no attempt was made to fractionate the collagen.

Quantitatively, collagen is the most important constituent of connective tissue, in which three forms of this protein are known to exist. One form, referred to as acid-soluble collagen, can be extracted either with dilute acetic acid (Zachariades, 1900; Nageotte, 1927; Leplat, 1933) or by acid buffers (Tustanovskii, 1947). The second form was isolated by Highberger, Gross & Schmitt (1951) from skin by extraction with slightly alkaline phosphate buffers and more recently with neutral salt solution (Gross, Highberger & Schmitt, 1955; Jackson & Fessler, 1955). This fraction has been called 'alkali-soluble collagen' (Harkness, Marko, Muir & Neuberger, 1954) or 'neutral-salt-soluble collagen' (Jackson & Fessler, 1955) and will be referred to in this communication by the latter name. The bulk of the collagen in connective tissue is insoluble but can be rendered soluble by transformation into gelatin.

It has been suggested that acid-soluble collagen is the precursor of insoluble collagen because its concentration in connective tissue decreased with age and because [α-^{14}C]glycine was incorporated more rapidly into acid-soluble collagen than into insoluble collagen (Orekhovitch, 1952, 1955). However, Harkness *et al.* (1954) showed that [α-^{14}C]glycine is incorporated most rapidly into collagen soluble in neutral salt solution, indicating that this was the true precursor, and further that insoluble collagen need not pass through the acid-soluble stage. It has also been suggested (Bowes, Elliott & Moss, 1953, 1955) that insoluble collagen is a complex of acid-soluble collagen and a muco-protein of high tyrosine content. That there is no sharp division between acid-soluble and insoluble collagen was indicated by the increased solubility in acid of tendon treated with hyaluronidase (Jackson, 1953).

Robertson & Schwartz (1953) reported that as much as 10 g. of relatively homogeneous tissue could be obtained by their technique, and it seemed likely that this offered a good opportunity to investigate the occurrence of the three collagen fractions enumerated above during the development of new connective tissue. The incorporation of [α-^{14}C]glycine into these fractions was also studied. Preliminary communications on part of this work have already been made (Jackson, 1956*a*, *b*).

EXPERIMENTAL

Treatment of animals. Male and female albino guinea pigs (National Institute for Medical Research stock) were used throughout. Their weights at the time of injection varied between 600 and 700 g. The animals were fed on diet no. 18 (Bruce & Parkes, 1946), hay and greens. Ascorbic acid was supplemented by giving orally, every other day, 50 mg. dissolved in 10% (w/v) sucrose as suggested by Robertson & Schwartz (1953).

A portion (5 ml.) of a 1% (w/v) solution of carrageenin in 0·9 (w/v) NaCl was injected subcutaneously into the lower abdominal region, under ether anaesthesia. After 5 days, 50 μc of [α-^{14}C]glycine in 1 ml. of 0·9% NaCl was injected intraperitoneally into each animal; 625 μc of $Na_2{}^{35}SO_4$ in 1 ml. of 0·9% (w/v) NaCl was also injected at the same time.

The animals were killed in groups of three by ether inhalation, and a 2 mm. strip was cut out longitudinally through the skin, new connective tissue (granuloma) and abdominal muscle, and fixed for histological examination. The skin was then peeled back from the ventral surface and the mass of new tissue separated from the abdominal and chest wall, macerated with scissors, weighed and divided for collagen and mucopolysaccharide studies. The results of the latter are reported in a separate communication (Slack, 1957).

Isolation of collagen fractions

All extractions were carried out at 2° with octan-2-ol as preservative. The main steps in the fractionation procedure are shown in Fig. 1.

Neutral-salt-soluble collagen. The macerated tissue was suspended in 10 times its weight of 0·2 M-NaCl (pH 7·4). The extraction was continued for 24 hr., with occasional stirring, and the mixture centrifuged for 30 min. at 1500 *g*. The supernatant was decanted through filter paper and re-centrifuged at 12 000 *g* for 30 min.

The procedure was repeated three times and the extracts were combined. Neutral-salt-soluble collagen was precipitated by addition of NaCl to a final concentration of 20% (w/v). The precipitate formed was removed by centrifuging at 12 000 *g* for 30 min. The clear supernatant was dialysed against frequent changes of water for several days, and insoluble material filtered off, and the solution made up to a known volume. A portion was freeze-dried and examined by filter-paper electrophoresis at pH 8·6 by the method of Durrum (1950). The remainder was evaporated to dryness, the residue being designated 'water-soluble protein' (Fig. 1, fraction 1). The solid obtained by salt precipitation was purified by the method previously described (Jackson & Fessler, 1955; Jackson, Neuberger, Leach & Jacobs, unpublished data) and the product was designated 'purified neutral-salt-soluble collagen' (Fig. 1, fraction 2*a*). The material normally discarded during purification was retained for determination of total neutral-salt-soluble collagen (Fig. 1, fraction 2*b*).

Acid-soluble collagen. To the residue remaining after extraction with 0·2 M-NaCl was added 10 times its volume of 0·2 M sodium citrate buffer, pH 3·5, and the extraction was continued for 24 hr. with occasional stirring. The mixture was centrifuged for 30 min. at 1500 *g* and filtered. The extraction was repeated three times and the extracts were combined and made up to a known volume. One-fifth of the total volume was evaporated to dryness and the residue used for determination of total acid-soluble collagen (Fig. 1, fraction 3*b*). To the remainder NaCl was added to a final concentration of 5% (w/v). The fibrous precipitate obtained was removed by centrifuging at 1500 *g* for 30 min. and redissolved in 0·01 N acetic acid, forming a clear viscous solution which was dialysed against water. The precipitate formed was further purified by repeating the procedure, the final precipitate being dried *in vacuo* over P_2O_5 and designated 'purified acid-soluble collagen' (Fig. 1, fraction 3*a*).

Insoluble collagen. The residue remaining after citrate extraction was extracted twice with 0·1 N-NaOH (Lowry, Gilligan & Katersky, 1941), and washed on the centrifuge three times with water. The material was then autoclaved twice at a pressure of 15 lb./in.^{2} in the presence of 25–50 ml. of water. The gelatin solutions obtained were combined and made up to a known volume. A measured fraction was taken and evaporated to dryness, the residue being used for determination of total insoluble collagen (Fig. 1, fraction 4*b*). The remainder was filtered hot through a clarifying filter (Ford's Sterimat F.C.B.). Trichloroacetic acid was added to a concentration of 5% (w/v) and the small amount of precipitate obtained after standing at 0° for several hours was removed by filtration. The clear filtrate was concentrated and poured into 10 vol. of acetone, allowed to stand at 0° overnight, and the precipitate was washed with ether and dried over P_2O_5. This material was designated 'purified insoluble collagen' (Fig. 1, fraction 4*a*).

The residue obtained after autoclaving was negligible and was discarded.

Chemical analysis

The collagen fractions were hydrolysed by heating in a sealed tube with 5·7 N-HCl for 24 hr. at 100° in refluxing water. The hydrolysates were filtered and evaporated to dryness *in vacuo* over P_2O_5 and solid NaOH. The hydrolysates of the fractions 1, 2*a*, 2*b*, 3*b*, 4*b* (Fig. 1) were dissolved in water and made up to a known volume. Samples (1 ml.) were used to determine the hydroxyproline content by the method of Neuman & Logan (1950). Collagen was estimated by assuming a value of 13·6% hydroxyproline for all the collagen fractions (Bowes *et al.* 1955). The remainder of fractions 1 and 2*a* and the hydrolysates from the purified fractions were used to isolate 2:4-dinitrophenylglycine for radioactivity measurements.

Isolation of 2:4-dinitrophenylglycine, and radioactivity measurements. The preparation and isolation of 2:4-dinitrophenylglycine was done by the method of Perrone (1951) as described by Neuberger, Perrone & Slack (1951). The solution of 2:4-dinitrophenylglycine obtained was made up to 25 or 50 ml. and the compound was estimated colorimetrically (Krȍl, 1952) with a Unicam spectrophotometer (wavelength 460 mμ.). The solution was evaporated to dryness

Fig. 1. Scheme of fractionation of connective tissue proteins. All operations were carried out at 2°.

Table 1. *Variation with time, after injection of carrageenin, of the concentration of the collagen fractions expressed in mg./100 g. wet wt. of tissue*

		mg./100 g. wet wt. of tissue				
			Neutral-soluble			
Time (days)	Wet wt. of tissue* (g.)	Total collagen	Total	Water-soluble	Acid-soluble	Insoluble
3	19·4	14·9	5·5	4·7	0·2	9·2
5	37·9	74·3	18·8	9·7	11·2	44·3
6	56·7	189·3	15·5	6·0	18·2	155·6
7	70·2	333·2	18·9	9·2	28·9	285·5
9	25·5	857·7	58·9	20·3	86·9	711·9
14	23·5	986·1	103·3	41·2	115·1	767·7
21	8·7	1068·3	159·0	58·5	155·2	754·1
28	4·7	717·0	136·5	55·0	148·6	431·9

* Total from three guinea pigs.

in vacuo and the radioactivity measured on solid samples of 'infinite thinness' as in the method of Henriques, Henriques & Neuberger (1955).

RESULTS

Development of the connective tissue

The development of the connective tissue following injection of carrageenin was similar to that described by Robertson & Schwartz (1953). The wet weight (Table 1) increased rapidly up to the seventh or ninth day and then fell quite rapidly until after 6 weeks nothing remained but a thin layer of fatty tissue. Histological examination showed that there was a steady infiltration of fibroblasts and other cells from the subcutaneous tissue and from the muscle wall into the layer of carrageenin gel formed after injection. Extracellular carrageenin disappeared after about 10 days. At 3 days no fibres were visible but from 5 days onwards increasing numbers of non-orientated fibres appeared, including a proportion of thin argyrophilic fibres. From about the fourteenth day until the tissue disappeared completely the fibrous material was gradually replaced by fat. A detailed account of the histological findings will be presented elsewhere (Williams, 1957).

Determination of collagen fractions

All three collagen fractions were found, namely: neutral-salt-soluble, acid-soluble and insoluble, and in addition a previously undescribed fraction containing hydroxyproline was found in the water-soluble proteins. The total collagen concentration (Table 1) increased slowly at first and more rapidly between the sixth and ninth days. Maximum concentration was reached at 14 days, after which the concentration fell off. However, since the wet weight decreased from days 7 to 9 onwards the absolute maximum amount of collagen was present about the ninth day.

Table 2. *Relative proportions of collagen fractions expressed as percentage of total collagen*

	Percentage of total collagen			
	Neutral-soluble			
Time (days)	Total	Water-soluble	Acid-soluble	Insoluble
3	38·4	30·7	1·6	60·0
5	25·3	13·0	15·0	59·7
6	8·4	3·3	9·6	82·0
7	4·5	2·2	6·9	88·6
9	6·8	2·3	10·1	83·1
14	10·5	4·2	11·7	77·8
21	14·9	5·4	14·5	70·6
28	19·0	7·7	20·7	60·3

At 3 days neutral-salt-soluble collagen appeared (Table 1). The concentration of this fraction increased up to the fifth day and remained constant for 2–4 days before rising again coincident with the decrease in the wet weight of tissue. At 3 days the major proportion of the neutral-salt-soluble collagen was not precipitated by salt or by dialysis. This water-soluble hydroxyproline-containing material was present throughout the period of the experiment, the concentration–time curve being parallel to that for the total neutral-salt-soluble collagen (Table 1). The concentration of acid-soluble collagen, although low at day 3, increased throughout the time of the experiment. The concentration of insoluble collagen also increased steadily up to 14 days, the most rapid increase being between the fifth and ninth day. After 14 days its concentration decreased steadily.

In Table 2 the results are expressed as percentage of total collagen, showing the relative proportions of the three collagen fractions. As insoluble collagen accumulates the relative proportions of the soluble collagens decrease until the weight of tissue begins to decrease, when an increasing proportion of the total collagen is found to be in a soluble form.

Isotope experiments

The highest specific activity was found in the neutral-salt-soluble collagen. Two hours after injection its activity was already high and reached a maximum between 8 and 12 hr. (Fig. 2). The activity had fallen rapidly by 24 hr. and more slowly thereafter, substantial activity still remaining after 16 days.

The activity of acid-soluble collagen was low at 2 hr. and rose rapidly to a maximum at 12 hr., considerably below the maximum activity of neutral-salt-soluble collagen. The activity–time curve for insoluble collagen was a little lower than that for acid collagen and reached a maximum after 24 hr. Activity had fallen after 48 hr., and after levelling out continued to fall from the fourth day on.

There was some variation in the activities of comparable fractions at comparable times, in experiments carried out at different times, but the activities of the different fractions relative to each other remained the same. Similar findings have been reported in the collagen fractions from rabbits injected intraperitoneally with [α-^{14}C]-glycine (Harkness *et al.* 1954).

DISCUSSION

The results presented here confirm the findings of Robertson & Schwartz (1953) that carrageenin injected into guinea pigs stimulates the formation of connective tissue, but differ in detail. These authors found that maximal amounts of tissue were obtained after 14 days and that resorption began after 3 weeks. In the present study maximal amounts of tissue were obtained after 7–9 days, resorption occurring immediately after the tissue had reached its maximum weight (Table 1).

The concentration–time curve of the total collagen content of the tissue (Table 2) is very similar to that reported by Dunphy & Udupa (1955) in healing wounds. These authors suggested that when only small amounts of collagen were produced, only the polysaccharide of the amorphous matrix and the soluble-collagen precursor were produced, the normal collagen fibres appearing rapidly thereafter. In regenerating tendon the concentration of collagen did not begin to rise until the eighth day and continued to rise steadily for over 28 days (Kodicek & Loewi, 1955).

Collagen fractions

The detailed analysis of the tissue (Fig. 1) showed the presence of all three collagen fractions known at present. Of the total collagen present on the third day 40 % was neutral-salt-soluble, practically the whole of the remainder being insoluble. Since no fibres were visible on histological examination, it is likely that no new insoluble collagen had been formed at this stage of development, the small amount found probably being derived from contamination with collagen from pre-existing loose fasciae. Thus it is probable that at this stage of development only neutral-salt-soluble collagen had been produced and that this fraction is the precursor of insoluble collagen as suggested by Harkness *et al.* (1954), since it is essentially the same fraction as the alkali-soluble collagen of these authors (Jackson & Fessler, 1955).

The finding that a proportion of the neutral-salt-soluble collagen was water-soluble was unexpected, since negligible amounts of hydroxyproline-containing material were found in the same fraction from the skins of young rabbits (Harkness *et al.* 1954; Jackson, unpublished results). The significance of this finding is not clear, but it is possible either that this material represents an earlier stage of collagen synthesis or that it is a lower-molecular-weight fraction of neutral-salt-soluble collagen which is known to aggregate in solution to varying degrees (Jackson & Fessler, 1955). The presence of haemoglobin makes it difficult to interpret the electrophoretic pattern and it is being further investigated.

The concentration of insoluble collagen increases with time up to 14 days. Since the wet weight of tissue decreases after the seventh to ninth day, i.e. the tissue is breaking down and is being reabsorbed, synthesis and breakdown must be taking place simultaneously at least from the seventh day. It is possible that both take place simultaneously from the beginning but that synthesis predominates at first and breakdown later. It has been found that carrageenin injected intradermally causes a rapid breakdown of the dermal-collagen fibres without preceding granuloma formation (Williams, 1957). The increase in concentration of both the soluble fractions which occurs concurrently with the decrease in wet weight suggests that the breakdown of insoluble collagen occurs first by a disaggregation into the units which make up mature collagen fibres producing acid and neutral-salt-soluble collagen, rather than by destruction of the constituent polypeptide chains, although this will probably follow disaggregation. However, in normal connective tissue development, the mature collagen fibres are stable and metabolically relatively inert (Neuberger *et al.* 1951). Hence the insoluble collagen produced by the method described here may be inherently unstable. However, in view of the results reported by Williams (1957) it seems more likely that carrageenin first stimulates the formation of normal insoluble collagen and then stimulates some mechanism which leads to its removal.

A comparison of the concentration–time curves of total bound sulphate (Slack, 1957) and total collagen shows that as the concentration of collagen increases, the concentration of bound sulphate decreases. This is compatible with histological evidence that during the development of new connective tissue there is first an accumulation of an amorphous matrix which, on the basis of its staining properties with toluidine blue, is believed to contain sulphated polysaccharides (Wolbach & Howe, 1926). As fibres appear, this matrix gradually disappears (Stearns, 1940*a*). Similar findings were reported by Dunphy & Udupa (1955), who compared the concentration–time curves for hexosamine and hydroxyproline in healing wounds.

Isotope experiments

The results are in complete agreement with the suggestion put forward by Harkness *et al.* (1954) that neutral-salt-soluble collagen and not acid collagen as suggested by Orekhovitch (1952) is the true precursor of insoluble collagen. The more recent work of Orekhovitch (1955), reiterating his belief that acid collagen is the true precursor, is invalidated because the method of extraction used would produce a mixture of the two soluble collagens; likewise the activity–time curve obtained would be the resultant of the curves for the two separate fractions.

Harkness *et al.* (1954) also suggested that acid-soluble collagen and insoluble collagen are not distinct groups of proteins but are both obtained from fibres deposited outside the cell. They also express the opinion that it is unnecessary for all the insoluble collagen to pass through the acid-soluble stage. The activity–time curves obtained in the present study support these ideas, since those of acid-soluble collagen and insoluble collagen are very similar and suggest that [α-^{14}C]glycine is incorporated simultaneously into both fractions.

There are some differences in detail between the present study and that of Harkness *et al.* (1954). The rate of incorporation of [α-^{14}C]glycine into the neutral-salt-soluble collagen is more rapid, maximum incorporation occurring at about 10 hr. as compared with 24 hr. for the same fraction from rabbit skin. The activity–time curves for acid-soluble collagen and insoluble collagen are also different in the two studies, maximum incorporation into acid-soluble collagen occurring in about 12 hr. as compared to 3 days for rabbit skin, and into insoluble collagen in 24 hr. as compared to 8 hr. These differences may be due to species difference or more probably to the higher rate of growth of the connective tissue stimulated by carrageenin.

Harkness *et al.* (1954) believed that the comparatively high activities which they obtained for insoluble collagen at 8 and 24 hr. were due to contamination with non-collagen protein of high activity. In the present study this difficulty did not arise, the activity of insoluble collagen being low at 2 hr., when the activity of the neutral-salt-soluble collagen was already very high (Fig. 2).

The persistence of radioactivity in the soluble collagens for as long as 16 days (Fig. 2) is probably due to the breakdown of insoluble collagen. If, as is suggested above, acid-soluble collagen and neutral-salt-soluble collagen are produced during breakdown, then these fractions would contain a component derived from insoluble collagen into which [α-^{14}C]glycine had been previously incorporated, and a component produced by synthesis involving inert glycine. The fact that the activity–time curves of all three collagens are very similar from the seventh to the twenty-eighth day after carrageenin injection would support this suggestion.

The morphological origin of insoluble collagen is well known, but the origin of the two soluble fractions is less clear. Harkness *et al.* (1954) suggest that acid-soluble collagen is derived from collagen recently laid down: the outer layers of larger fibres and the thin fibrils which are prevalent in developing connective tissue (Gross, 1950) and which are argyrophilic and known as reticulin. Preliminary experiments (Jackson & Williams, unpublished results) involving histological examination of connective tissue stimulated by carrageenin after the various stages of extraction (Fig. 1) have been carried out. After extraction with 0·2 M-NaCl, the thin argyrophil fibres no longer took up silver, but stained red with the van Gieson stain as do the thick mature fibres. Thus the argyrophil properties of these thin fibrils may be due to a coating of neutral-salt-soluble collagen not yet incorporated into the fibre structure, although

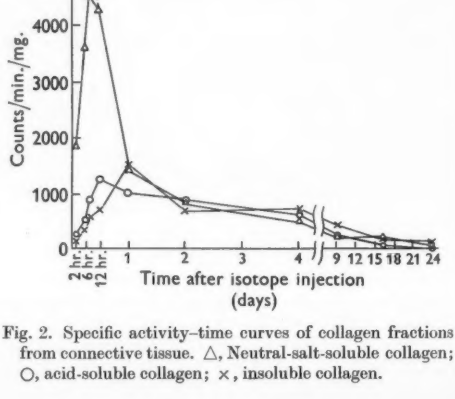

Fig. 2. Specific activity–time curves of collagen fractions from connective tissue. △, Neutral-salt-soluble collagen; ○, acid-soluble collagen; ×, insoluble collagen.

other proteins which are removed by neutral-salt solutions, could be the responsible factors. After citrate extraction the argyrophil fibres were almost completely absent, suggesting that these are in fact acid-soluble collagen and hence different from basement-membrane reticulin (Kramer & Little, 1953; Windrum, Kent & Eastoe, 1955). The absence of microscopically visible fibres 3 days after carrageenin injection, when neutral-salt-soluble collagen is present (Table 1), suggests that this fraction also occurs in the non-fibrillar part of connective tissue.

It cannot be decided from the present study whether any of the neutral-salt-soluble collagen isolated was originally intracellular. Studies by Porter & Vanamee (1949), using the combined techniques of tissue culture and electron microscopy, have shown that early fibrils are formed at the cell surface, and may be clearly connected with intracellular fibroglial fibres. These early fibrils reach a mature form extracellularly by accretion of material from the ground substance.

These findings have been confirmed (Jackson, 1954*a*, *b*) in tissue culture of embryonic bone. It was not possible to decide whether the fibroglial fibres were actually intracellular (Jackson & Smith, 1955). However, collagen fibres fail to develop in tissue culture in the presence of anti-collagen serum (Robbins, Watson, Pappas & Porter, 1955), forming instead amorphous masses having the tinctorial properties of collagen; these are probably antigen–antibody complexes. This would suggest that a non-fibrous precursor is secreted by the cell, forming fibrous collagen extracellularly. Cytoplasmic granules in connective tissue cells have been put forward as possible precursors of both amorphous matrix and collagen fibres (Jackson & Smith, 1955). Stearns (1940*a*, *b*) concluded on the basis of her observations of fibre formation in a transparent chamber in the rabbit ear that these granules secreted a soluble precursor into the extracellular space, where this material was transformed into a fibrous form. It has recently been shown that neutral-salt-soluble collagen can precipitate spontaneously into typical collagen fibres (Jackson & Fessler, 1955). It would seem probable that intracellular collagen is non-fibrous and is secreted into the extracellular spaces, from which it can be extracted as a neutral-salt-soluble collagen.

On the evidence available a tentative scheme for the formation of collagen in developing connective tissue may be put forward. Neutral-salt-soluble collagen (or an intracellular precursor) is secreted into the extracellular space by the fibroblast. This type of collagen is then transformed into fibrillar collagen in various ways:

(1) By forming new fibrils which are argyrophilic, which behave as acid-soluble collagen.

(2) These fibrils accumulate more neutral-salt-soluble collagen to increase their length and diameter and become insoluble collagen. Banfield (1955) has shown in electron-microscope studies in normal developing connective tissue and in granulation tissue, that the width and length of collagen fibres increase with age.

(3) By direct incorporation into pre-existing insoluble collagen fibres, further increasing their length and diameter.

The first two ways would be the most important in the early stages of development, whereas the last would predominate in the later stages.

The reason for the final insolubility of mature collagen is still obscure. Bowes *et al.* (1953, 1955) have suggested that it is due to the interaction of acid-soluble collagen with a non-collagenous protein. Recently these authors have suggested that this protein may even form an integral part of the collagen fibres (Bowes, Elliott & Moss, 1956). Since neutral-salt-soluble collagen is probably the precursor and can apparently add on directly to insoluble collagen, the latter suggestion seems more likely. The increasing insolubility of collagen with age might then be due to increased aggregation of collagen molecules, as was suggested by Harkness *et al.* (1954).

Despite the complexity introduced by the breakdown of the connective tissue formed under the stimulus of carrageenin, the phenomenon described would appear to offer a basis for studies of the factors which influence the formation of the various components of connective tissue and, what may be more important from the pathological point of view, the factors influencing its breakdown, connective tissue breakdown being a feature of many connective tissue diseases.

SUMMARY

1. Subcutaneous injection of carrageenin into guinea pigs stimulates the formation of new connective tissue which is resorbed after 4–6 weeks.

2. The change in concentration with time of neutral-salt-soluble collagen, acid-soluble collagen and insoluble collagen, and the incorporation of [α-^{14}C]glycine into these collagens, have been studied.

3. Maximum wet weight of new tissue is produced between the seventh and ninth day and disappears after 4–6 weeks, although the total concentration of collagen in the tissue does not reach a maximum until the fourteenth day.

4. Most of the insoluble collagen is formed between the fifth and ninth day. Neutral-salt-soluble collagen appears before acid-soluble collagen, the concentration increasing up to the fifth day, remaining constant and then increasing rapidly as the tissue is resorbed. The concentration of acid-

soluble collagen increases throughout the experimental period.

5. The highest specific activity is found in neutral-salt-soluble collagen, maximum activity occurring at 10 hr., with a half-life of 18–20 hr. The activity–time curves of acid-soluble collagen and insoluble collagen are similar, maximum activity occurring at 12 and 24 hr. respectively and being very much lower than the activity of neutral-salt-soluble collagen.

6. It is concluded that neutral-salt-soluble collagen is the precursor of collagen fibres and can either be transformed into acid-soluble collagen or add on to pre-existing acid-soluble collagen and insoluble collagen.

7. A water-soluble protein containing hydroxyproline has also been found.

I am indebted to Dr G. Williams for the histological examination of the tissues and to Mr J. P. Bentley for his valuable technical assistance. The carrageenin was the gift of Mr L. Stoloff of Seaweed Chemicals Inc., New Brunswick, to whom I express my thanks.

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The Stabilization of Purified Human Albumin to Heat

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Scatchard, Gibson, Woodruff, Batchelder & Brown (1944) recorded changes in the turbidity and viscosity caused by heating solutions of purified human albumin at temperatures up to 57°. They found that at pH 6·8 a 25% solution of albumin in 0·3M sodium chloride could be heated at 57° for 10 hr. without demonstrable change in turbidity. The clinical need for albumin solutions low in salt concentration which could be sterilized by heat led to the studies of Ballou, Boyer, Luck & Luen (1944) on the effect of organic ions on the thermal stability of albumin. They found, using nephelometric methods, that caprylate, phenylacetate and acetyltryptophan improved the thermal stability of

albumin. Since Brand, Kassell & Saidel (1944) had found that human albumin contained only 0·19 % of tryptophan, nutritional considerations influenced the adoption as a standard for the albumin issued for clinical use in the U.S.A. of a 25 % solution of the Harvard Fraction V stabilized at pH 6·8 with 0·04 M sodium acetyltryptophan. This solution could be heated for 10 hr. at 60° without alteration in viscosity or turbidity.

Solutions of albumin prepared by the method of Kekwick & Mackay (1954) and sterilized by irradiation with ultraviolet light were found to be satisfactory for transfusion (Martin, 1954). When Murphy (1953) demonstrated that this technique could not be relied on to destroy the serum hepatitis virus, albumin solutions of 6·25 % protein concentration were heated at 58° for 10 hr. instead of being irradiated, a procedure which Gellis *et al.* (1948) claimed would destroy the virus of hepatitis. No gross change in viscosity or in osmotic pressure was observed after the heat treatment and no untoward effects were observed when the heated material was transfused. However, both electrophoretic and ultracentrifugal analysis suggested that some modification of the albumin had taken place and, since no data could be found in the literature, it was decided to examine electrophoretically and in the ultracentrifuge the effect of controlled heating on albumin, and the modification of these effects resulting from the prior addition of amino acids and their acetyl derivatives to the albumin solutions examined.

MATERIALS AND METHODS

Albumin. The albumin used was fraction AP 2 (Kekwick & Mackay, 1954), the material normally supplied for intravenous use. One batch (LA 582) was used for all the experiments. It contained 97 % of albumin and 3 % of β-globulin, as estimated by electrophoresis, and moved as a single component in the ultracentrifuge ($S^0_{20,w}=4{\cdot}20$ for a 1 % solution). This material had been dried from the frozen state from aqueous solution and was reconstituted in 0·1 M-$NaHCO_3$ to a protein concentration of 10 % (w/v).

Amino acids. Solutions (0·1 M) of glycine, valine, tyrosine, tryptophan and phenylalanine in 0·15 M-NaCl, and 0·1 M solutions of acetylglycine, acetylvaline, acetyltyrosine, acetyltryptophan and acetylphenylalanine in 0·15 M-NaCl, were used. The purity of each amino acid was checked by chromatography.

Viscosity measurements. These were carried out in an Ostwald viscosimeter at 37°, and the flow time of the heated material was compared with that of the unheated albumin and with water. The flow time of the standard viscosimeter used was 14 sec. for water at 37°.

Electrophoretic measurements. (*a*) Moving-boundary electrophoresis: samples were dialysed to equilibrium at 2° against sodium phosphate buffer, pH 8·0, *I* 0·2, and diluted with buffer to a concentration of 1·5 % protein. Measurements were made in the Tiselius (1937) apparatus at 0° with a potential gradient of 6 v/cm. Optical observations by the diagonal-schlieren method (Philpot, 1938) were photographically recorded with monochromatic light (546 mμ.). The photograph was projected with an enlargement of eight diameters and tracings were made. The curves were analysed by reflexion across median lines to determine the quantities of the components present. The results are expressed as the percentage of total protein in a mixture attributable to any single component. The analytical values quoted are the mean from a pair of exposures obtained simultaneously from ascending and descending limbs.

(*b*) Electrophoresis on paper: analysis of samples was carried out in the apparatus described by Franglen, Martin & Treherne (1955) with a protein concentration of 3·4 % in sodium diethylbarbiturate buffer, pH 8·6, *I* 0·06, on Whatman no. 1 paper at a potential difference of 3·5 v/cm. for 16 hr. The proteins were stained with bromophenol blue, and the protein concentrations estimated by the elution technique of Cremer & Tiselius (1950), 0·02 N-$NaHCO_3$ being used in a Unicam spectrophotometer with a 1 mm. cell and a wavelength of 595–600 mμ. (Franglen & Martin, 1954). Results are shown in graphical form and are accurate to ± 4 %. Since the dye binding of the modified albumin might differ from that of the native material, the eluted fluid from four pairs of results was analysed for N content, and the ratios of the two components were compared with those obtained colorimetrically. There was no significant difference in the results obtained by the estimation of N and those obtained colorimetrically.

Ultracentrifuge measurements. Dialysed samples were diluted to give a concentration of 1 % protein so that the solution contained phosphate buffer, pH 8·0, *I* 0·2, in 0·15 M-NaCl. The solutions were subjected to 250 000 *g* in the Svedberg oil-turbine ultracentrifuge, a 12 mm. cell being used. The optical recording was the same as that described for electrophoresis.

EXPERIMENTAL AND RESULTS

Effects of time and temperature of heating. Preliminary experiments were carried out on dilute solutions. Albumin solutions [10 % (w/v) protein at pH 6·5] were diluted with 0·15 M-NaCl to a protein concentration of 3·4 %, and 6 ml. portions placed in thin-walled tubes closed with rubber stoppers. The tubes were immersed in a thermostat, the temperature of which was controlled to $\pm 0{\cdot}4°$. Samples of the material were withdrawn at intervals, immediately cooled to 2° and stored at this temperature, together with an unheated sample, until the heating of all samples had been completed. Albumin solutions held at 43°, 51°, 53°, 56°, 58° and 60° for periods up to 24 hr. were examined by electrophoresis on paper and by viscosity measurements, and compared with the unheated sample. The results of the electrophoretic analyses are shown in Fig. 1, in which the percentage of unaltered albumin as judged by the analysis is plotted against the time of heating.

Viscosity measurements on these samples showed a slight but progressive increase in the flow time compared with unheated albumin (19·2–22·0 sec.,

at 58°; 17 sec. when unheated). At protein concentrations above 6·25 % the albumin will gel at 58° in 1 hr. See also Scatchard *et al.* (1944).

Effects of amino acids on the changes produced by heat. Albumin was diluted with 0·15 M-NaCl, and then with solutions of the amino acids listed above dissolved in 0·15 M-NaCl, so that the final protein concentration was 3·4 % and the amino acid concentration was 20 mg./g. of protein. As the previous experiments had established that modification had occurred after heating the albumin to 58° for 1 hr., these conditions were used in the experiment. The degree of modification was estimated by electrophoresis on paper. None of the amino acids used prevented the modification of albumin except acetyltryptophan and, to a lesser degree, acetylphenylalanine.

Quantities of acetyltryptophan needed to stabilize albumin. Ballou *et al.* (1944) had shown that a 25 % (w/v) solution of albumin in 0·04 M acetyltryptophan (40 mg./g. of protein) would withstand a temperature of 60° for 10 hr., and our own preliminary experiments showed that 20 mg./g. would stabilize albumin dissolved in 0·15 M-NaCl. The action of a range of concentrations of acetyltryptophan in a more concentrated albumin solution was then tried. To 10 % (w/v) albumin solution, in 0·1 M-$NaHCO_3$, acetyltryptophan was added to a final concentration of 1, 3 or 5 mg./g. of protein. The solutions were heated to 58° for 1 hr. and examined by electrophoresis on paper. This showed that there was gross modification of the albumin by heat at the lower acetyltryptophan concentrations, and only a slight modification at 5 mg./g. of protein. Albumin solutions containing 5, 10 and 20 mg. of acetyltryptophan/g. of protein (10 % albumin in 0·1 M-$NaHCO_3$) were then heated for 6 hr. at 58°, since these conditions approximated to those used in the preparation of albumin for transfusion. The heated albumin was examined by moving-boundary electrophoresis, and in the ultracentrifuge. Samples of heated albumin containing 1, 3 and 5 mg./g. of acetyltryptophan were also examined in the ultracentrifuge. Results of the electrophoretic examinations are shown in Table 1 and indicate that there was some modification at 5 mg./g. of protein, very slight modification at 10 mg./g. of protein and none at 20 mg./g. of protein.

No alteration in the ultracentrifuge analyses was detected at any concentration of amino acid, including those which had shown modification by both of the electrophoretic techniques.

A solution of albumin (5 g. of protein/100 ml. of 0·15 M-NaCl) containing 10 mg. of acetyltryptophan/g. of protein was heated at 58° for 24 hr. Samples examined at intervals showed no modification for 7 hr., but showed progressive changes from 7 to 24 hr. (Fig. 2).

The effects of heat on albumin in solution at pH 6·8 and with acetyltryptophan and tryptophan are shown in Fig. 3.

Effect of heat on conjugation of albumin with 1-anilinonaphthalene-8-sulphonic acid. Samples of a 3·4 % solution of albumin in 0·15 M-NaCl, pH 6·8, were coupled with 1-anilinonaphthalene-8-sulphonic acid, as described by Weber & Laurence (1954), before and after heating at 58° for 2 hr. with and without acetyltryptophan. The faster-moving

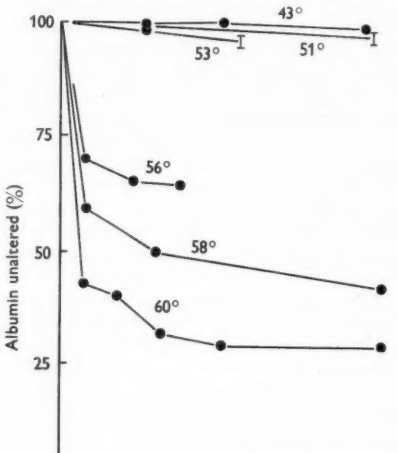

Fig. 1. Effect of heat on a solution of albumin. Protein concentration: 3·4 g./100 ml. Solvent: 0·15 M aqueous solution of sodium chloride, pH 6·8. Ordinates represent the percentage of total albumin surviving after the period of heating shown on the abscissa, computed from electrophoretic analysis. I Indicates boundary spreading but no clearly defined second component.

Table 1. *Effect of acetyltryptophan on heat stability of albumin*

Solutions of albumin, with the exception of the control, which was unheated, were heated at 58° for 8 hr.

	Electrophoretic analysis (% total protein)		
Acetyltryptophan (mg./g. of protein)	Albumin first component	Albumin second component	Globulin
0	41·5	53·3	1·2
5	84·2	12·2	3·6
5	84·6	12·7	2·7
10	95·5	1·5	3·0
20	97	0·0	3·0
Control	97	0·0	3·0

electrophoretic component regularly produced a fluorescent conjugate similar to that described by Weber & Laurence, whereas the slower component, which appears as the result of controlled heating of albumin in the absence of acetyltryptophan, failed to produce a fluorescent conjugate with the dye.

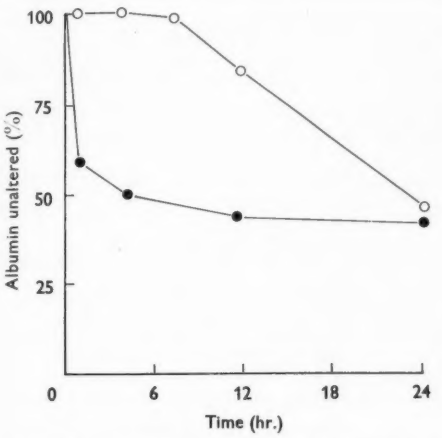

Fig. 2. Effect of prolonged heat on a solution of albumin with and without acetyltryptophan. Protein concentration: 5·0 g./100 ml. Solvent: 0·15 M aqueous solution of sodium chloride, pH 6·8. ○, Acetyltryptophan 10 mg./g. of protein; ●, no acetyltryptophan.

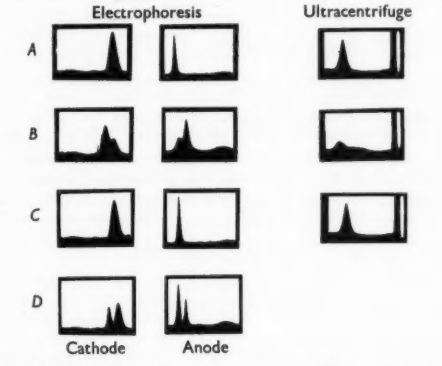

Fig. 3. Electrophoretic and ultracentrifugal diagrams showing the effects of heat on purified human albumin. Electrophoresis: 15 mA for 3 hr. in buffer, pH 8·0, *I* 0·2. Ultracentrifuge: 250 000 *g* for 1 hr. in buffer, pH 8·0, *I* 0·15. *A*, Unheated albumin; *B*, albumin heated at 58° for 8 hr.; *C*, albumin heated at 58° for 8 hr. with 20 mg. of acetyltryptophan/g. of albumin; *D*, albumin heated at 58° for 1 hr. with 20 mg. of tryptophan/g. of albumin.

DISCUSSION

The use of human albumin as a therapeutic agent makes it essential that every precaution should be taken to prevent the possible transmission of virus infection. Since doubts had been cast on the efficiency of ultraviolet light as a practical means of sterilization it was necessary to re-examine the effect of heat on albumin at concentrations likely to be used for transfusion and at temperatures which have some sterilizing effect.

Our earliest investigations indicated that albumin submitted to temperatures of the order of 58° for more than 1 hr. undergoes some modification, as judged by electrophoresis. Practical experience showed that no harmful effects resulted from the transfusion of the material (Kekwick & Mackay, 1954), but it was obvious that a procedure resulting in a loss of at least 30 % of the primary albumin must be wasteful. Moreover, it cannot be assumed that the 'second component', the result of heating, is an adequate substitute for native albumin.

From our experiments it is seen that controlled heat affects albumin in two stages; there is first the reduction in total net charge observed by electrophoretic analysis. This change is associated with a qualitative change as illustrated by the failure of the second component to associate with 1-anilinonaphthalene-8-sulphonic acid to produce a fluorescent compound. These modifications are observed under conditions which do not produce demonstrable alterations in the ultracentrifugal pattern or gross changes in viscosity. Higher temperatures or prolonged heat result in a change in the ultracentrifugal pattern, which, taken in conjunction with the subsequent changes in viscosity and, finally, actual increase in nephelometric reading, suggest molecular aggregation producing a polydisperse system.

The temperature–time curves also show two distinct phases: the first, almost complete in the first hour, associated with the rapid production of component 2, and the second, a slower change, continuing over the next 24 hr.

The presence of acetyltryptophan appears to modify but not inhibit the first molecular reorientation at concentrations equivalent to three molecules of acetyltryptophan/molecule of albumin.

Tryptophan has no comparable effect at any concentration we have investigated. The effect of acetylation of the 2-amino group may alter the capacity of the carboxyl group of tryptophan to combine with the available basic groups of the albumin.

If heating results in cross-linking involving basic groups it would be reasonable to suppose that acetyltryptophan is effective by blocking cross-linkage. We have no direct evidence which would help to identify the precise groups in the albumin molecule which are involved.

SUMMARY

1. Purified human albumin heated in solution in 0·15 M sodium chloride at pH 6·8 develops a second component.

2. At temperatures of 45° and 50° prolonged heating is required to modify albumin, but at temperatures from 56° to 60° the modification is established in 1 hr.

3. In dilute solution slight but progressive increases in viscosity were noted at 58° if heating was prolonged. Solutions of 5–6·25% could be heated without gross change in viscosity, but above this protein concentration albumin gels.

4. The action of a number of amino acids and acetyl amino acids on the heat stability of albumin was examined, but all were ineffective except acetyltryptophan, and to a lesser degree acetylphenylalanine.

5. The stabilizing effects of different concentrations of acetyltryptophan were estimated. Solutions containing 20 mg. of acetyltryptophan/g. of albumin may be heated for 10 hr. at 60° without modification of the protein.

6. Moving-boundary electrophoresis proved more sensitive in detecting modification than electrophoresis on paper, the ultracentrifuge, or changes in viscosity.

We would like to thank Dr J. R. Laurence for a sample of 1-anilinonaphthalene-8-sulphonic acid.

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The Speed of Several Cerebral Reactions Involving the Nicotinamide Coenzymes

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A recent survey of respiration in cerebral systems (McIlwain, 1955) showed that although there is qualitative evidence and general expectation that the greater part of cerebral respiration involves nicotinamide coenzymes and cytochromes, known rates of individual reactions oxidizing the reduced coenzymes are too low to account for the oxygen consumption of the brain or of isolated cerebral tissues. Thus respiration of rat or guinea-pig cerebral cortex with glucose and other substrates can reach 120 or 150 μmoles of O_2/g. of tissue/hr.; but the tissue has been observed to reduce by reduced cozymase only 220 μmoles of cytochrome *c*/g./hr., corresponding to 55 μmoles of oxygen/g./hr.

To bridge this gap we have investigated the rates at which cerebral tissues catalyse a number of relevant reactions. These have included oxidation of reduced cozymase by cytochrome *c* and by air, and the corresponding reactions of reduced 2′-phosphocozymase. Interconversion of the two coenzymes has been sought, and also optimum conditions for oxidation of their reduced forms by glutathione.

EXPERIMENTAL

Materials

Cozymase (of 90–95% purity), cytochrome *c* (salt-free preparation of approx. 90% purity) and adenosine triphosphate were from the Sigma Chemical Co. and from Boehringer, Mannheim. Ethylenediaminetetraacetic acid (EDTA) was from Eastman Organic Chemicals.

Reduced cozymase and 2′-phosphocozymase. Specimens of approx. 90% purity were obtained from the Sigma Chemical Co. In addition, solutions of the reduced coenzymes were prepared as follows (see Shuster & Kaplan, 1955; Asnis, 1955). Sodium dithionite (15 mg.) in the stopper of an evacuated Thunberg tube was tipped promptly into 20 mg.

of $NaHCO_3$ and 7 mg. of coenzyme in 2 ml. of water. After 10 min. at 37°, the tube was cooled and opened and O_2 passed vigorously for 5 min. at room temp. The solutions (0·20–0·05 ml. in 3 ml. of water) were assayed absorptiometrically and kept at −20°. For studying glutathione reductase these solutions were not adequate, apparently because they contained materials derived from the dithionite, and the Sigma specimens were used.

In examining the possible interaction of cytochrome *c* and the reduced nucleotides, the following control experiments were carried out. The preparation of reduced nucleotide was incubated with the cytochrome *c* reductase reaction mixture without added tissue, and its absorption at 550 mμ. observed during 10 min. No change was found, nor was change observed in a similar mixture in which the reduced coenzyme solution was replaced by a 'blank' solution made by incubating dithionite and bicarbonate and then oxygenating. Also, the 'blank' solution was incubated in the complete cytochrome reductase mixture with tissue, and no change in absorption observed during 20 min. at 37°. Change at approx. 300 μmoles of cytochrome/g. of tissue/hr. was, however, observed when reduced cozymase was added.

Glutathione. Reduced and oxidized forms were purchased from Light and Co. In addition, the oxidized form was prepared by Rall & Lehninger's (1952) procedure but without attempting to free it from solvent of crystallization; it had an $[\alpha]_D^{12}$ of −97°, suggesting that the specimen retained some 10% of solvent. This was confirmed by its equivalence in oxidation of reduced 2′-phosphocozymase.

Tissue preparations. Organs were removed promptly, weighed and put into ice-cold test-tube homogenizers with 9 vol. of cold water or of 0·25 M sucrose and ground. The suspensions were kept at 0–3° and further dilutions made immediately before use. The part designated brain stem lay ventral to the cerebellum of the guinea pig or rat, and consisted largely of medulla and pons. Cerebral hemispheres consisted of cortical grey and subcortical white matter, without lower structures.

Acetone-dried powders, except in the instance noted below, were made by cooling the tissue in a glass-homogenizer tube, adding 7 vol. of acetone at 0°, and rapidly homogenizing and centrifuging. The supernatant liquid was discarded, the tissue residue re-extracted twice more with acetone as before, and the residue dried *in vacuo*.

In studying the phosphorylation of cozymase the following preparations, based on those of Wang & Kaplan (1954), were employed. In different experiments the brains of guinea pigs and sheep were rapidly ground in 20 vol. of ice-cold acetone, the residue twice further treated with acetone, and dried *in vacuo*. The acetone-dried powders were extracted, in some cases twice, with 11·5 ml. of cold 0·02 M-$KHCO_3$/g. of powder. Protamine sulphate [0·2% in 0·04 M aminotrishydroxymethylmethane (tris), pH 7·5] was added with stirring to the extract (Wang & Kaplan, 1954), precipitates being collected after addition of 8 and 10 ml. of the protamine solution and extracted with 0·2 M acetate buffer, pH 5. Preparations were also made from the $KHCO_3$ extract by precipitating with the 0·2 M acetate buffer.

Methods

A number of preliminary experiments, not described in detail, were performed to ensure that conditions for the estimations were optimum.

Cytochrome c reductase. Conditions initially were based on those of Hogeboom (1949), Mahler, Sarkar, Vernon & Alberty (1952) and Brody, Wang & Bain (1952); the reaction mixture concentrations were 33 mM sodium phosphate, pH 7·4, 0·2 mM-KCN, 35 μM cytochrome *c*, 67 or 100 μM reduced nicotinamide nucleotide and an appropriate tissue preparation. It was found advantageous to adopt Slater's (1950) procedure and mix first the components other than cyanide and reduced coenzyme in spectrophotometer cells in the thermostat, and to leave these together for 10–15 min. for temperature equilibration and for the cytochrome to be completely oxidized by the dissolved O_2 of the solutions.

The reaction was normally followed by a change in absorption at 550 mμ. on reducing the cytochrome. In preliminary experiments the visible spectrum of the reaction mixture was recorded and the bands characteristic of reduced cytochrome *c* were observed to appear. Normally 8–12 readings were taken at intervals of 0·5–1·5 min., according to the speed of the change and the rate calculated from the initial linear group of values; this included at least six readings. In certain cases the change at 340 mμ., due mainly to oxidation of the reduced nucleotide, was followed. Allowance was then made for the concomitant change in cytochrome absorption as described by Hogeboom (1949). Stoicheiometric relationships were demonstrated between the main reactants by adding 4·8 μm-moles of reduced cozymase, which was found to produce a change in extinction at 550 mμ. corresponding to 10 μm-moles of cytochrome *c*.

At 23°, reactants in the concentrations described were demonstrated to be in excess: the initial reaction rate with reduced cozymase at 30 μM was the same as that at 60 μM. With cytochrome *c* at 15 μM it was the same as at 35 μM. Relations at 37° are described below.

Oxidation of reduced nicotinamide nucleotides by air. Reaction mixtures normally contained reagents in the following final concentrations: 33 mM sodium phosphates, pH 7·4, 0·3 mM EDTA, 5 μM cytochrome *c*, 150 μM reduced nicotinamide and a suitable dilution of a tissue preparation. Reagents were added in the order named to sufficient water to give a final volume of 3 ml. in two spectrophotometer cells. A third cell contained the mixture but without nucleotide. Absorption at 340 mμ. in the first two with the third as control was measured during a 10 min. period at 37° to allow temperature equilibration before adding the tissue; it was found to change little if at all during this period. A suitable dilution of a tissue preparation was then added to the first two cells and measurements were made each 0·5–2 min. The solution as ordinarily handled would be expected to be 220 μM with respect to dissolved oxygen from the air, or to contain over four times the amount necessary to oxidize all the reduced nucleotide.

Systems converting cozymase into 2′-phosphocozymase. Evidence for this conversion by cerebral tissues was sought under the conditions in which it had been measured in liver by Wang & Kaplan (1954).

The preparations from brain described above, and also some made from liver by the same procedure, were assayed in a reaction mixture containing reagents in the following concentrations: 6·7 μM cozymase, 8 mM adenosine triphosphate, 10 mM-$MgCl_2$, 100 mM tris, pH 7·5, and 10 mM nicotinamide. In some cases synthesis was examined with one-quarter of these concentrations of reagents, and in

others nicotinamide was omitted. Reaction mixtures (0·5 ml.) were incubated for times between 10 and 120 min. and deproteinized by heating. 2′-Phosphocozymase was then determined with *iso*citric dehydrogenase of pig heart by the method of Ochoa & Weisz-Tabori (1948) as modified by McIlwain & Rodnight (1949). It was confirmed that liver extracts were capable of the conversion, rates of 0·5 μmole of 2′-phosphocozymase formed/g. of tissue/hr. being observed. Small quantities (0·1 μmole) of 2′-phosphocozymase added to the cerebral reaction mixtures were recovered.

Glutathione reductase. Initial conditions were based on those of Rall & Lehninger (1952) and Asnis (1955); the concentrations of reagents in the reaction mixture were 50 mM sodium and potassium phosphates, pH 7, 0·5 mM oxidized glutathione, 36 μM reduced 2′-phosphocozymase and an appropriate tissue preparation. These were later modified as described in the Results section.

Absorptiometric measurements. These were made with a Hilger Uvispek mark II photoelectric spectrophotometer. The cell compartment was fitted with a thermometer and a water jacket through which water from a thermostatically controlled bath was circulated. Quartz cells of 1 cm. light path were employed, some taking 3 ml., and others 0·23 ml., of fluid. The change in extinction coefficient at 550 mμ. on reducing cytochrome *c* was taken from the data of Haas, Horecker & Hogness (1940) and of Slater (1950) as 18·5 cm.^{2}/μmole. The change at 340 mμ. on oxidizing reduced cozymase and its 2′-phosphate was taken as 6·22 cm.^{2}/μmole (Horecker & Kornberg, 1953).

RESULTS

Cytochrome c reductases

Reduction of cytochrome *c* by reduced cozymase was initially measured under conditions described by previous investigators (see above) and proceeded in suspensions from guinea-pig brain at 100–300 μmoles/g./hr. (Fig. 1; Table 1). Rates in different experiments varied considerably and fell if the tissue was left for a few minutes at 37° before reaction was commenced by adding reduced cozymase. Added substances were included in the reaction mixture as possible activating or stabilizing agents. Glutathione, which could itself reduce cyctochrome *c* in this reaction mixture, did not lead to higher rates, nor did glycylglycine; but serum albumin, and especially EDTA, did so. This acid has been used by Bonner (1954) in the succinic oxidase system of heart. Rates between 400 and 1000 μmoles of cytochrome *c* reduced/g. of tissue/hr. were then obtained for different parts of the brain, and with liver some 2500 μmoles were reduced/g./hr.

For maximum rates it was found necessary to prepare the tissue suspension in sucrose, and to carry out any dilution in sucrose just before the assay. With sucrose suspensions in reaction mixtures containing EDTA, areas of the brain stem proved to be of high and reproducible activity in reducing cytochrome *c* by reduced cozymase, and with such preparations conditions for optimum activity were further explored. The activity was not significantly altered when the EDTA concentration was varied between 0·13 and 1·33 mM, and a concentration of 0·33 mM was adopted. When phosphates were used as the main inorganic component in reaction mixtures, highest rates were obtained with solu-

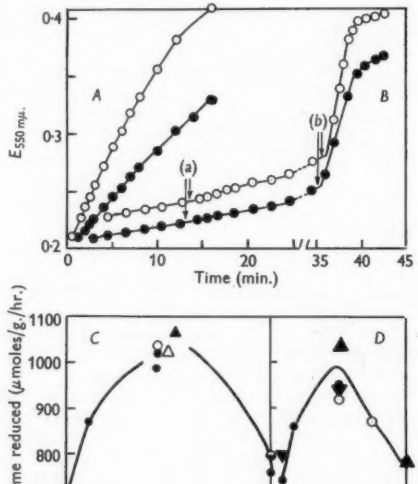

Fig. 1. Reduction of cytochrome *c* by reduced nicotinamide coenzymes. *A*, Change measured in the reaction mixture of Table 1, but with 0·3 mM EDTA and 0·25 mg. (○) and 0·125 mg. (●) of guinea-pig midbrain/3 ml. *B*, Change measured in the reaction mixture of Table 1, with (○) and without (●) mM EDTA and with 0·625 mg. of guinea-pig midbrain and 10 μmoles of reduced 2′-phosphocozymase present initially/3 ml. A further 50 μm-moles of the phosphocozymase were added at (*a*) and 50 μm-moles of reduced cozymase at (*b*). *C*, Rate of reduction of cytochrome by reduced cozymase in the reaction mixture of Table 1 containing 0·3 mM EDTA, but in which the inorganic constituents were altered as follows: ●, Phosphate molarity altered as indicated on the abscissa; ○, phosphate replaced by NaCl; ◒, equimolar phosphate and glycylglycine; △, 13 mM phosphate and 33 mM-NaCl; ▲, 5·5 mM phosphate and 50 mM-NaCl. In expressing concentrations in osmolar units the phosphates were assumed to yield 3 equiv./mole and the NaCl 2 equiv. Rates were derived from experiments such as that of Fig. 1*A* with guinea-pig midbrain. *D*, Effect of phosphate buffers (33 mM) of different pH on the reduction in the reaction mixture of Table 1, by reduced cozymase. The differently shaped points refer to different preparations of guinea-pig brain stem.

Table 1. *Cytochrome c reductase of guinea-pig tissues*

Reaction mixtures contained 33 mM sodium phosphate, pH 7·4, 35 μM cytochrome *c*, 0·2 mM-KCN, 0·1 mM reduced coenzymes and 0·1–0·2 mg. of tissue/ml. Rates quoted are the mean of two values agreeing to within 5%, except when followed by standard deviation and, in parenthesis, the number of experiments. When two values are quoted they refer to different animals. Rates with glutathione were corrected for rate of reduction in the absence of reduced cozymase.

Tissue	Suspension fluid	Additions to reaction mixture	Rate of cytochrome *c* reduction (μmoles/g./hr.) By reduced cozymase	By reduced 2′-phospho-cozymase
Whole brain	Water	None	115*, 120*	—
		None	272±11 (4)	15
Brain stem	0·25 M Sucrose	None	470±16 (6)	20
		EDTA, 0·33 mM	883±24 (6)	12, 16
		Plasma albumin, 1%	560	—
		Glutathione, 2 mM	463	—
Liver	Water	None	730, 750	—
	0·25 M Sucrose	EDTA, 0·33 mM	2460, 2540	—

* At 22°.

tions about 30 mM in phosphate, which is the concentration recommended by previous workers but is markedly hypotonic (Fig. 1*C*). It could be replaced by NaCl of similar tonicity (50 mM), and a little phosphate added to the NaCl had little effect on the rate (no attempt has been made to render the tissue preparation deficient in phosphate). A pH of 7·4 proved to be optimum (Fig. 1*D*), again in agreement with findings with other tissues. The concentration of reduced cozymase in the normal reaction mixture (67–100 μM) appeared to give optimum rate, as indicated by the linear course of the greater part of the reaction curves of Fig. 1*A*. It was, however, important to investigate this directly, and results are given in Fig. 2*A*. Here the rate is seen to reach its maximum at about 100 μM cozymase; half-maximal rate was at some 20 μM.

Each part of the brain examined was also capable of slowly reducing cytochrome *c* in presence of 2′-phosphocozymase. The rate (Table 1) averaged about 5% of that with cozymase, when the reduced phosphocozymase was examined at 0·02–0·1 mM. The presence of 0·06 mM reduced phosphocozymase, did not affect the rate of reduction of cytochrome *c* by reduced cozymase added later in the same concentration although a change in rate of 10% would have been detectable. No information has been obtained which might indicate whether a single system oxidizes the two coenzymes.

Nicotinamide nucleotide oxidases

Reduced cozymase was relatively slowly oxidized when added to ground cerebral tissues in salt mixtures (Table 2). Rates were found of some 30 μmoles oxidized/g. of brain stem/hr. in comparison with an observed respiratory rate of up to 100 μmoles of O_2 absorbed/g./hr., suggesting the simple conditions to be inadequate. Findings just

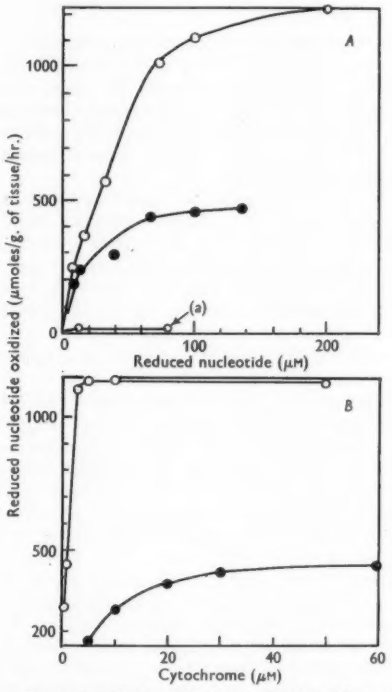

Fig. 2. Rate of oxidation of reduced nicotinamide nucleotides (cozymase except at *a*, when phosphocozymase was used) by air (○) and by cytochrome *c* (●). *A*, Dependence of the rates on the concentration of reduced nucleotides. *B*, Dependence on cytochrome *c*. Reaction mixtures for reduction of cytochrome *c* by reduced nucleotides as in Fig. 1*A* except where indicated; for oxidation of reduced cozymase, the full reaction mixture of Table 2.

Table 2. *Requirements for oxidation of reduced cozymase with oxygen by cerebral suspensions*

The complete reaction mixture contained 33 mM phosphate, pH 7·4, 33 μM EDTA, 5 μM cytochrome *c* and 150 μM reduced cozymase. Tissues were ground in 0·25 M sucrose and used at 0·1–0·2 mg./ml. Mean, standard deviation and number of experiments are given in three cases; other values are averages of duplicates agreeing to within 10%.

Tissue	Reaction mixture	Loss of reduced cozymase (μmoles/g./hr.)
Brain stem, guinea pig	Complete	781±27 (6)
	No cytochrome	114
	No cytochrome, no EDTA	29
	No cytochrome, no EDTA; with 10 mM histidine	115
	No EDTA	400
	No EDTA; with 10 mM histidine	730
Brain stem, rat	Complete	1056±34 (6)
	With 0·1 or mM adenosine diphosphate	1010
	No cytochrome	92
	No cytochrome; with 0·3 mM adenosine diphosphate	108
	No EDTA	270
	No EDTA; with 0·3 mM adenosine diphosphate	290
Cerebral cortex, rat	Complete	616±28 (4)

described in relation to cytochrome reductases, and also those of Slater (1950) and Bonner (1954) on oxidation of reduced cozymase by other tissues, prompted the addition of cytochrome *c*, adenosine diphosphate, inorganic phosphate, histidine and EDTA. With certain of these additions, rates were increased 20- to 40-fold.

Two main requirements were found: for cytochrome *c*, and for either histidine or EDTA (Table 2). Relatively little EDTA was found necessary; rates with 330 and 33 μM were similar and the latter concentration was adopted for further work. It could be replaced by 10 mM histidine. Requirements for cytochrome *c* by the oxidase system were much lower than those of cytochrome reductase; 5 μM gave the same rate as 50 μM and half-maximal rate was given at about 0·6 μM concentration of cytochrome *c* (Fig. 2*B*). This concentration, although low, corresponds to a turnover rate of only some 200 moles of cozymase formed/mole of cytochrome/hr. Values of similar magnitude can be derived from Slater's (1950) data for oxidation of reduced cozymase by kidney preparations.

The reaction proved to be maximal at about pH 7·4 (Fig. 3). It showed a requirement for inorganic salts which was met by NaCl or K_2SO_4 (Table 3), which afforded higher reaction rates than did glycylglycine. Beyond this general requirement two ions were found to have specific effects: phosphate in accelerating the reaction and calcium salts in depressing it. The lower rate with 1·4 mM-$CaCl_2$ was similar to that found in the absence of EDTA. The presence of lower concentrations of calcium salts, 0·08 and 0·24 mM, which were below that of the EDTA (0·3 mM, equivalent to 0·6 mM Ca^{2+}), had little effect on the reaction.

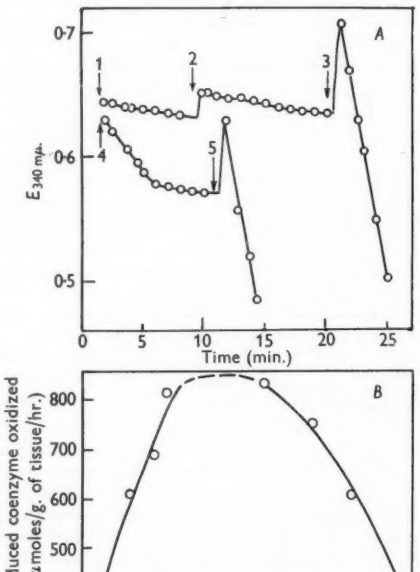

Fig. 3. Oxidation of reduced nicotinamide nucleotides with oxygen by suspensions of brain stem. *A*, Rat tissue, 0·33 mg./ml. with full reaction mixture of Table 2 except for nucleotides, which were added as follows: at 1, 16·7 μM, and at 2, 100 μM phosphocozymase; at 3 and 5, 100 μM, and at 4, 16·7 μM reduced cozymase. *B*, Full reaction mixture of Table 2 with 33 mM phosphate buffers of the pH indicated; guinea-pig medulla ground in sucrose was added at 0·33 mg./ml.

Table 3. *Oxidation of reduced cozymase: effect of inorganic salts*

Experiments were with rat brain stem suspended in sucrose and (except when otherwise indicated) added to the full reaction mixture of Table 2, with 0·3 mM EDTA. Concentrations of constituents listed were chosen to give 96–106 osmolar solutions, assuming the phosphate and K_2SO_4 to yield 3 equiv. and KCl 2 equiv.

Constituents of media (mM)			Rate of oxidation (μmoles of cozymase/g./hr.)
Phosphates	NaCl	Others	
33	0	0	1090
10	33	0	1130
25	13	0	1060
0	50	0	805
0	13	Glycylglycine, 80	565
0	0	Glycylglycine, 100	450
0	0	K_2SO_4, 33	815
30	0	KCl, 2·7; $MgSO_4$, 0·7	990
30	0	KCl, 2·7; $MgSO_4$, 0·7; $CaCl_2$, 1·4	560
30	0	KCl, 2·7; $MgSO_4$, 0·7; $CaCl_2$, 0·08 or 0·24	1030

Cozymase and phosphocozymase. The reaction mixture based on the findings of the preceding paragraphs contained cytochrome c and EDTA and was buffered with phosphates at pH 7·4. In it, appreciable concentrations of reduced cozymase were found to be needed for optimum reaction rate (Fig. 2*A*), half-maximal oxidation being reached with about 40 μM reduced cozymase. Reaction with reduced 2′-phosphocozymase was much slower, averaging with brain stem 20–30 μmoles/g./hr. (Fig. 2). This was obtained with low concentrations of phosphocozymase, of no more than 10 μM. Reduced cozymase, added to suspensions subsequently to 20 or 80 μM reduced phosphocozymase, was oxidized at normal rate.

Glutathione reductase

Oxidation of reduced 2′-phosphocozymase by cerebral as by other tissues has been found to be greatly accelerated by addition of oxidized glutathione, which underwent concomitant reduction (Fig. 4; see Vennesland & Conn, 1954). Conditions for assay of the reductase were developed from those of Rall & Lehninger (1952), but the preparation of the tissue and the reaction mixture were found to need more detailed examination.

Extraction. Though liver ground in sucrose was described as of the same reductase activity as an extract of an acetone-dried powder of the tissue (Rall & Lehninger, 1952) this was not found to be the case with cerebral tissues. Preparations in which the tissue was ground in sucrose were found of lower activity than others ground in water, and these of lower activity than extracts of acetone-dried powder. Other manipulations were therefore examined in searching for conditions in which the tissue exhibited maximal activity (Fig. 4*B*). Keeping the tissue suspension in 9 vol. of water at 2–3° led to a slow increase in activity; greater

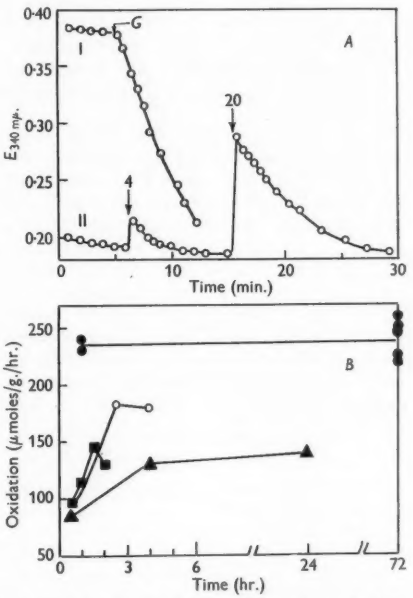

Fig. 4. Glutathione reduction by guinea-pig cerebral tissue with reduced 2′-phosphocozymase in the full reaction mixture quoted on p. 294, except as specified. *A*, Phosphate extract of acetone-dried cerebral hemisphere with: I, 83 μM reduced nucleotide present initially and 300 μM glutathione added at *G* (0·3 subtracted from all ordinates); II, with initially 300 μM glutathione and no added nucleotide; concentrations indicated (μM) were added as shown. *B*, Rate of oxidation of the reduced nucleotide by different preparations (see text) of guinea-pig cerebral hemispheres. ●, Acetone-dried material, extracted with phosphate, pH 7, and the extract kept at 2° for the time indicated. Tissue ground in water, and then kept: ▲, at 2° alone; ○, at 2° with *iso*butanol; ■, at 37° alone.

increase was obtained on keeping at 37° and again greater at 2–3° with *iso*butanol. These levels remained below that of acetone-dried powder extracted with either water or phosphate (50 mM, pH 7). Preparations in aqueous ethanol also gave lower rates. Activities both of the acetone-dried powders and of extracts from them were stable for several days (probably for longer) and were therefore accepted as a basis for assay.

Reaction mixture and conditions of assay. In unfractionated extracts of guinea-pig cerebral hemispheres, glutathione reductase has not proved exacting in its requirement for inorganic salts. The 50 mM sodium phosphates used previously could be largely replaced by mixtures of glycylglycine and NaCl (Table 4); replacement of the NaCl by Na_2SO_4, or its omission, gave lower rates. EDTA increased rates by about 60 % and was included in the standard reaction mixture. The following materials had little effect on reaction rate: 0·3 mM cysteine, 0·1 mM $MnCl_2$ and a crude specimen of flavine–adenine dinucleotide. The reaction was 25 % faster at pH 7 than at pH 8, and 15 % faster at pH 7 than at pH 6.

On this basis the concentrations of reagents in the full reaction mixture used in the following experiments were 50 mM sodium and potassium phosphates at pH 7, 330 μM EDTA, 83 μM reduced 2′-phosphocozymase, 300 μM oxidized glutathione and a preparation equivalent to 0·1–5 mg. of tissue/ml.

With the normal excess of oxidized glutathione, added limited quantities of reduced coenzyme were found to be almost quantitatively removed (Fig. 4*A*). With excess of reduced coenzyme, limited quantities of oxidized glutathione gave a change in extinction showing that almost 1 equiv. of reduced coenzyme had been removed, actual quantities being: added, 45 μmoles; removed, 41, 45 μmoles. When in this mixture the concentration of reduced phosphocozymase was decreased, marked dependence of reaction rate on the coenzyme was observed at levels below 20 μM (Fig. 5). When the oxidized glutathione was varied in level, concentrations below 250 μM were associated with lower rates (Fig. 5). The average rate observed with preparations from guinea-pig cerebral hemispheres

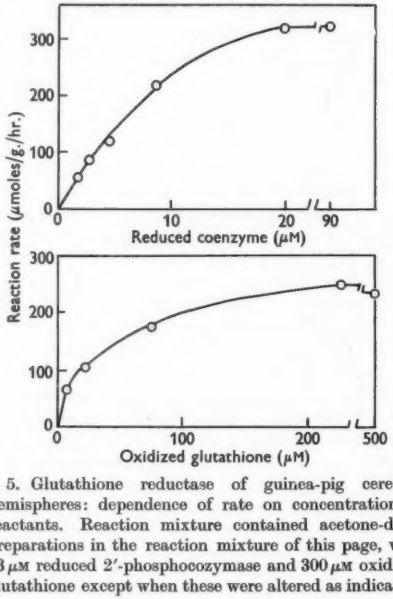

Fig. 5. Glutathione reductase of guinea-pig cerebral hemispheres: dependence of rate on concentration of reactants. Reaction mixture contained acetone-dried preparations in the reaction mixture of this page, with 83 μM reduced 2′-phosphocozymase and 300 μM oxidized glutathione except when these were altered as indicated.

Table 4. *Glutathione reductase of guinea-pig cerebral tissues and of liver*

Results are expressed as in Table 2. Full reaction mixture is described in the text above.

Preparation	Departure from full reaction mixture (mM)	Rate (μmoles of reduced 2′-phosphocozymase lost/hr./g. of tissue)
Cerebral hemisphere		
A. Phosphate extract of acetone-dried powder	None	240±9 (5)
	No EDTA	150
	Phosphates, 0·4; glycylglycine, 100	175*, 190
	Phosphates, 0·4; glycylglycine, 33; NaCl, 33	250*
	Phosphates, 0·4; glycylglycine, 33; K_2SO_4, 22	250*
B. Ground in water; used immediately	None	98
	No EDTA	56
C. Preparation *B* after keeping at 2° for 24 hr.	None	140
	$MgSO_4$, 0·2; KH_2PO_4, 0·2; KCl, 1; NaCl, 21	105
Liver	None	980

* The further addition of 3·3 mM sodium phosphate, pH 7, made no change in these rates.

in the full reaction mixture was 250 μmoles of coenzyme reacting/g. of fresh tissue/hr., or some six times that previously reported in rat brain (Rall & Lehninger, 1952).

Other reactions

Conversion of cozymase into 2′-phosphocozymase has been sought, as described in the experimental part, but the rate of any conversion appeared to be less than 0·1 μmole/g./hr. Under the conditions in which reduced 2′-phosphocozymase was oxidized by cytochrome *c* or by air, any rapid dephosphorylation would have been shown, but was not detected; whether the slow oxidation occurring was an immediate oxidation of the 2′-phospho compound or was secondary to other changes, has not been determined.

DISCUSSION

Metabolic routes concerning cozymase and 2′-phosphocozymase in cerebral tissues are thus relatively distinct. Interconversion of the coenzymes by phosphorylation of cozymase was much slower than in liver; the main oxidation of reduced cozymase through cytochromes proceeded much more rapidly than did the corresponding reaction in phosphocozymase; and an oxidation corresponding to that of reduced phosphocozymase by glutathione scarcely proceeded with reduced cozymase.

Cozymase. The previous discrepancy (see Introduction) between rates of respiration in cerebral tissues and their rates of oxidation of reduced cozymase has received its explanation largely in terms of methods of measuring the oxidation, rather than in terms of alternative reactions in the coenzymes. Reduced cozymase was oxidized in a mixture containing inorganic phosphate, added cytochrome *c*, and EDTA or histidine, at some 600 μmoles/g. of cerebral cortex/hr. This, equivalent to 300 μmoles of oxygen/g./hr., is approximately twice the greatest rate of absorption of oxygen which has been observed in cerebral systems *in vivo* or *in vitro* (see McIlwain, 1955). The rate of 600 μmoles/g./hr. was reached in tissue suspensions at pH values and at salt levels corresponding to those of the intact tissue. It may, however, be limited in the intact tissue by the available reduced cozymase. Although the total cozymase of rat brain is about 300 μm-moles/g., on the average more than one-half of this is oxidized, leaving some 130 $\pm$ 60 μm-moles/g. in the reduced form (Glock & McLean, 1955). Thus (see Fig. 2*A*), in some specimens the concentration of reduced cozymase may be adequate for only one-half of the maximal rate of oxidation. This computation and also later ones do not make allowance for the extracellular volume of the tissue, nor for any binding of cozymase, factors which would affect the available concentration of coenzyme in opposing senses.

Estimation of the rate of oxidation of reduced cozymase by cytochrome *c* in the mixtures containing cyanide appears to give a measure of only part of the potential activity of cytochrome reductase. From the rate of the oxidation of reduced cozymase by oxygen, accelerated by cytochrome *c* (600 μmoles of coenzyme/g./hr.), reduction of cytochrome *c* at 1200 μmoles/g./hr. must be involved. Rates actually observed, though higher than those of Brody *et al.* (1952) (220 μmoles/g./hr.), were, in cerebral cortex, about 400 μmoles/g./hr. That this is lower than 1200 is presumably due to difficulty of access of a succession of cytochrome *c* molecules to the enzyme during their reduction; a succession which may not be necessary when the cytochrome is an intermediary carrier rather than a major reactant. The concentration of cytochrome *c* found necessary in the oxidation by air was 3 μM (Fig. 2) in distinction to some 30 μM for the lower maximal rate reached in the reductase system. Previous observations supporting relatively high values for the native cytochrome reductase can be deduced from data of Wang & Bain (1953) and of Abood, Gerard, Banks & Tschirgi (1952), who found for reactions in rat brain in which cytochrome reductase was probably involved, rates corresponding to about 800 μmoles of coenzyme oxidized/g./hr. and 400 μmoles/g./hr. respectively.

2′-Phosphocozymase. Oxidation of the reduced phosphocozymase by cytochrome in the mixture containing cyanide reached some 10 μmoles of coenzyme/g. of tissue/hr., again about one-half of that of its oxidation by oxygen. Such oxidation could not account for more than 15 % of the tissue's maximal oxygen uptake. The quantity of 2′-phosphocozymase in rat brain is also relatively small (Gore, Ibbott & McIlwain, 1950): some 12 μm-moles/g., almost all in the reduced form (Glock & McLean, 1955). Related to this are two aspects of the present findings: first, in a 12 μM solution, reduced phosphocozymase is already being oxidized by oxygen at the maximal rate (Fig. 2*B*). Further, the rate of oxidation of reduced phosphocozymase by oxygen is some 2–5 % of the rate of oxidation of reduced cozymase, just as the quantity of phosphocozymase in the tissue is some 2–5 % of the quantity of cozymase.

The present search for optimum conditions for displaying the glutathione reductase of cerebral tissues has emphasized that this system forms the major route for oxidation of reduced 2′-phosphocozymase. Rates now observed reach 250–300 μmoles of glutathione or coenzyme reacting/g. fresh wt. of tissue/hr., or six times those earlier recorded. They thus correspond to 125–150 μmoles of oxygen/g./hr., which is greater than the normal rate of

respiration of the brain *in vivo* or *in vitro*, and falls not far short of maximal stimulated rates. Moreover, rates approaching the maximal can be reached with levels of reduced coenzyme and oxidized glutathione which exist in the brain. Considering the reduced coenzyme, the native level of 12 ± 4 μm-moles/g., if regarded as equivalent to a 12 ± 4 μM solution, could support oxidation at 180–300 μmoles/g./hr. (Fig. 5). Normally, however, this reaction is probably limited by the concentration of oxidized glutathione in the brain, which may be very low as it is in several organs of the body (Bhattacharya, Robson & Stewart, 1955). Fujita & Numata (1938) found the total glutathione in rat brain to be 2·7 μmoles/g., without detectable oxidized form, probably implying this to be less than 0·05 μmole/g. Kudryavtseva & Kudryavtseva (1950), however, suggest that up to one-fifth of the cerebral glutathione may be in the oxidized form in the brain of young rabbits. As the present findings indicate about 80 μM oxidized glutathione to be needed for half-maximal rate of the reductase, the different estimates give very different impressions of the rate at which the reductase is likely to proceed *in vivo*, and the levels are being re-examined.

Rates reported for systems reducing 2′-phosphocozymase in rat brain are: glucose 6-phosphate dehydrogenase, 20 μmoles/g./hr.; breakdown of ribose 5-phosphate, 45 μmoles/g./hr. (Glock & McLean, 1954). Successive operation of the systems could thus produce reduced phosphocozymase at 40 μmoles/g./hr. Regarding the remainder of a maximal respiratory rate of 150 μmoles of oxygen/g./hr. (i.e. 130 μmoles/g./hr.) as proceeding through the tricarboxylic cycle, when one-sixth of the oxidation is via *iso*citric dehydrogenase which reduces phosphocozymase, a further 43 μmoles/g./hr. of the reduced coenzyme could be formed, giving a total of 83 μmoles/g./hr. Systems now observed to remove the reduced coenzyme operate maximally at over three times this rate ($250 + 20$ μmoles/g./hr.). Thus the preponderance of reduced forms of the coenzyme and of glutathione in the tissue implies relatively slow oxidation of reduced glutathione. Rates of reactions oxidizing reduced cozymase can similarly be computed to be greater than those which form it. In accordance with this, the tissue's cozymase is found to be predominantly in the oxidized form.

SUMMARY

1. Optimum conditions for determining cytochrome *c* reductase in cerebral tissues involved their grinding in sucrose, the inclusion of ethylenediaminetetraacetate, of reduced cozymase at or above 100 μM and of cytochrome *c* at or above 30 μM. Up to 900 μmoles of cytochrome could then be reduced/g. of tissue/hr.

2. Oxidation of reduced cozymase by air also required ethylenediaminetetraacetate or a surrogate, reduced cozymase at or above 100 μM and added cytochrome *c* at or above 5 μM. It then proceeded at up to 1050 μmoles/g./hr.

3. Reduced 2′-phosphocozymase was oxidized only slowly in the preceding mixtures, but much more rapidly with added oxidized glutathione, in the presence of extracts of acetone-dried tissue, when rates up to 250 μmoles/g. of tissue/hr. were observed.

4. Processes interconverting the two coenzymes or their two reduced forms were relatively slow.

5. The rates of oxidation quoted are many times those previously recorded and are collectively much greater than those of the oxygen uptake of intact cerebral tissues *in vivo* or *in vitro*. Concentrations of the reduced coenzymes and of glutathione which occur in cerebral tissues approach but are not usually in excess of the minimal needed for maximal oxidation.

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The Biochemistry of the Nitrifying Organisms

5. NITRITE OXIDATION BY *NITROBACTER**

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Nitrobacter is an autotrophic soil bacterium that derives its entire energy supply from the reaction

$$NO_2^- + \tfrac{1}{2}O_2 = NO_3^- + 17 \text{ kcal.}$$

Apart from the early studies of Meyerhof (1916*a*, *b*), there has been little biochemical work done on this organism since its discovery and isolation by Winogradsky (1890, 1891). The present paper records investigations into the mechanism of nitrite oxidation by *Nitrobacter*. Preliminary notes on some of our results have already appeared (Lees & Simpson, 1955*a*, *b*).

MATERIALS AND METHODS

Growth of the organisms. *Nitrobacter* was obtained from an Aberdeen garden soil by serial subculture in a medium made up as follows. To 10 l. of water were added 30 g. of KH_2PO_4, 1 g. of $CaSO_4$, 0·5 g. of $MgCl_2$, 0·01 g. of $MnCl_2$, 0·2 ml. of dialysed iron (B.P.). By addition of 10M-NaOH, the pH was adjusted to 7·8, which is the optimum pH for the strain of *Nitrobacter* used (Lees, 1954). The solution was filtered and, after addition of the appropriate amount of $NaNO_2$, sterilized at 120° for 20 min. This medium (medium G) was used also for the propagation of the stock cultures which provided inocula for large-scale batch cultures. For large-scale batch cultures 100 ml. of the carbonate–phosphate suspension, used by Lees (1952) for the culture of *Nitrosomonas*, was added to each 10 l. of medium G before sterilization. Batch cultures were used for preparing suspensions of *Nitrobacter* cells suitable for biochemical study; the carbonate–phosphate suspension was added to facilitate the separation of these cells after growth. *Nitrobacter*, unlike *Nitrosomonas*, does not require a solid surface for growth, but *Nitrobacter* cells did adhere in some measure to the solid particles in the suspension and, in any case, were entrained by them when the cells were separated from the bulk of the medium by centrifuging.

The cells were grown at 28°. Stock cultures were carried in conical flasks; the depth of the medium was always less than 1 cm., to ensure adequate aeration. Batch cultures were grown in 10 l. of medium in 10 l. bottles, each fitted with a glass sparger through which air was pumped at about 10 l./min. The air was sterilized by passing it through 0·1M-$CuSO_4$ in N-H_2SO_4, then through a sterilized cotton-wool plug. A fresh stock, or batch, culture was initiated by inoculating the sterilized culture medium (containing 70 μg. of N/ml., as $NaNO_2$) with about 1% of its volume of stock culture. Samples of the newly inoculated culture were taken each day and analysed for nitrite. After all the nitrite initially added had been oxidized, twice the original amount was added. After this had been oxidized, four times the original amount was added, and so on. Nitrite oxidation in both stock and batch cultures usually ceased after about 3 weeks, when a total of about 1·4 mg. of nitrite N/ml. had been oxidized. Since washed suspensions of *Nitrobacter* cells prepared from batch cultures that had oxidized 1·4 mg. of nitrite N/ml. were capable of oxidizing nitrite vigorously in the Warburg apparatus, the inability of cultures to oxidize more than 1·4 mg. of nitrite N/ml. was presumably connected with the accumulation of nitrate or other metabolic end-products or both in the culture medium.

Separation of cells from batch cultures. After a batch culture had oxidized about 1 mg. of nitrite N/ml., the *Nitrobacter* cells were reaped as follows. (i) The culture was centrifuged in a Sharples Centrifuge (Sharples Centrifuges Ltd., Stroud, Glos.). The liquid passing through the centrifuge was discarded and the solid, containing all the cells, was suspended in 100 ml. of water. (ii) The suspension was agitated for 3 min. in a rotating-blade household homogenizer to detach the cells from the solid particles. (iii) The suspension was then made to about 300 ml. and centrifuged for 10 min. at 700 *g*. This treatment precipitated the solid particles, but left the cells in suspension. The supernatant fluid was poured off and the solid particles were again agitated and centrifuged in the same way. (iv) The combined supernatant fluids from the two centrifugings were then centrifuged at 11 000 *g* for 10 min.; this treatment precipitated the cells as a compact yellow-brown mass. The cells were washed once with water and stored at 0° in the buffer solution used almost universally throughout this work, i.e. 0·03M-KH_2PO_4 brought to pH 7·8 by addition of 10M-NaOH. The yield of cells from 10 l. of batch culture was about 200 mg. dry wt.

Purity of the Nitrobacter *cultures.* The cultures used were not pure, but were always contaminated by a single heterotrophic organism (apparently a pink yeast) that presumably grew on organic compounds made by *Nitrobacter*. Numerous attempts to purify the cultures failed. These attempts included plating the cultures on to nitrite–agar and on to nitrite–silica-gel; colonies of *Nitrobacter* that grew on these media always showed the presence of the heterotroph when transferred to nutrient agar on which the heterotroph, but not the *Nitrobacter*, would grow. Attempts to purify the *Nitrobacter* by dilution, or by treatment of the cultures with tetracycline, an antibiotic to which *Nitrobacter* seems to be largely insensitive, were equally unsuccessful. We thus had to use impure cultures of *Nitrobacter* for our work. The degree of contamination of the cultures was measured in two different ways. (1) The ratio of *Nitrobacter* cells to heterotrophic cells in washed-cell suspensions was estimated by

* Part 4: Hofman & Lees (1953).

direct microscopical count; the *Nitrobacter* cells were distinguished from the heterotrophic cells partly by the greater resistance of the *Nitrobacter* cells to staining (Gibbs, 1919), partly by their smaller size. (2) The number of *Nitrobacter* cells in a culture that had oxidized a given amount of nitrite was calculated on the twin assumptions that a *Nitrobacter* cell had a dry wt. of about 10^{-13} g. and that the free-energy efficiency of *Nitrobacter* was about 5% (Baas-Becking & Parks, 1927); the number of heterotrophs in the washed-cell preparation of *Nitrobacter* from the same culture was measured by plating the preparation on to nutrient agar. According to both methods of measurement, the washed-cell preparations of *Nitrobacter* from batch cultures, used throughout the present work, never contained more than 1 heterotrophic cell : 50 *Nitrobacter* cells. It therefore seemed reasonable to assume that these preparations were suitable for investigations of the biochemical aspects of nitrite oxidation.

Measurement of nitrite oxidation. This was usually followed manometrically in the Warburg apparatus at 25°. Owing to the fact that the Q_{O_2} (µl. of O_2/mg. dry wt. of cells/hr.) for *Nitrobacter* in the presence of nitrite was 100–150, only about 3 mg. dry wt. of cells was required for each vessel. The cells were suspended in 2·5 ml. of 0·03 M phosphate buffer, pH 7·8, and placed in the main vessel. Additions of $NaNO_2$ were made in 0·5 ml. of solution from the side arm some 10 min. later, after temperature equilibration of the Warburg vessels. In a few experiments nitrite oxidation was followed by measurements of the amount of nitrite that disappeared when a solution of $NaNO_2$ was incubated with *Nitrobacter*. Nitrite was estimated by a Griess–Ilosva technique (Lees & Quastel, 1946). Checks were also run from time to time on the amount of nitrate produced when a known amount of nitrite had been oxidized. These checks were based on a colorimetric estimation of nitrate by phenol-disulphonic acid (Lees, 1952).

Detection of cytochrome bands and measurement of their optical densities. The absorption bands of reduced cytochromes in *Nitrobacter* suspensions were detected, and their wavelengths roughly measured, by means of a Hartridge reversion spectroscope. The wavelengths and optical densities of the bands were precisely determined in a spectrophotometer. Difference spectra of the reduced and oxidized cytochrome components of *Nitrobacter* were obtained by comparing, at regular wavelength intervals, the optical density of a washed, well-aerated *Nitrobacter* suspension with that of a similar suspension treated with dithionite. The spectroscopic data for Fig. 8 were obtained by measuring the optical density of the *Nitrobacter* suspensions reduced by dithionite at 551 and 589 mµ.; for all measurements the same sheet of white paper, having a white-light transmission roughly equal to that of the cell suspensions, was used as a 'blank'.

The cytochrome most intimately concerned with nitrite oxidation in *Nitrobacter* was apparently a cytochrome that, in its reduced state, showed an absorption band at 551 mµ. (see 'Results'). This cytochrome will be referred to as 'cytochrome 551' in general; its reduced form will be designated $Fe^{2+}_{cyt.\,551}$, and its oxidized form $Fe^{3+}_{cyt.\,551}$.

Pre-incubation. In some experiments, *Nitrobacter* cells were pre-incubated with various compounds for different periods before being studied in the Warburg apparatus. The cells were suspended in 25 ml. of 0·03 M phosphate buffer, pH 7·8, containing, where appropriate, the compound under investigation. The pre-incubations were carried out at 25° in 100 ml. stoppered conical flasks; the flasks were usually shaken during incubation. From time to time a sample of 1–3 ml. was withdrawn from the flasks and run on to crushed ice in a 50 ml. polythene centrifuge tube which was immediately placed in the refrigerator at 0°. When all necessary samples had been taken, the tubes were removed from the refrigerator and exposed to room temperature until nearly all the ice had melted. The contents of each tube were then made to 50 ml. with ice-cold water and centrifuged at 0° for 10 min. at 11 000 *g* to separate the cells. Cells were resuspended in a measured volume of 0·03 M phosphate buffer, pH 7·8.

Anaerobic incubations were carried out in Thunberg tubes under N_2 at 25°.

Growth experiments. In these experiments, which were designed to test the effect of different compounds on the growth of *Nitrobacter*, 25 ml. lots of medium G containing 70 µg. of nitrite N/ml. as $NaNO_2$ (and, where appropriate, the compound under investigation) were sterilized in 100 ml. conical flasks at 120° for 20 min. and inoculated with a single loopful (approx. 0·01 ml.) of *Nitrobacter* culture. The flasks were then incubated at 28° and tested daily for the presence of nitrite. When the nitrite concentration of a culture had fallen below about 10 µg. of nitrite N/ml., growth of *Nitrobacter* cells in the culture was presumed to have taken place and the time between inoculation and presumed growth was recorded.

Sodium cyanate was prepared from a commercial sample by repeated crystallization from boiling methanol–water (9:1, v/v).

Nitrourea was prepared by the method of Davis & Blanchard (1929).

Sodium chlorite was prepared according to Partington (1939). The purity of the final sample, estimated by the amount of I_2 liberated when the chlorite was added to a solution of excess KI, was about 85%.

All other reagents, with the exceptions of the dithionite and fluoroacetate, were of AnalaR quality.

RESULTS

The oxygen uptake due to nitrite oxidation by *Nitrobacter* suspensions proceeded at a constant rate until almost all the nitrite had been oxidized; the rate then fell sharply almost to zero (Fig. 1). The oxygen uptake by *Nitrobacter* suspensions in the absence of nitrite was negligible (about 0·1 µmole of O_2/vessel/hr.). The rate of oxygen uptake varied with the initial nitrite concentration (Fig. 2). Estimations of nitrate carried out from time to time on the contents of experimental Warburg flasks showed that the amount of nitrite oxidized to nitrate always agreed, to within 5%, with the amount of oxygen consumed, according to the equation

$$NO_2^- + \tfrac{1}{2}O_2 = NO_3^-.$$

Agreement between oxygen uptake and nitrite oxidation is therefore to be inferred even where it is not specifically mentioned.

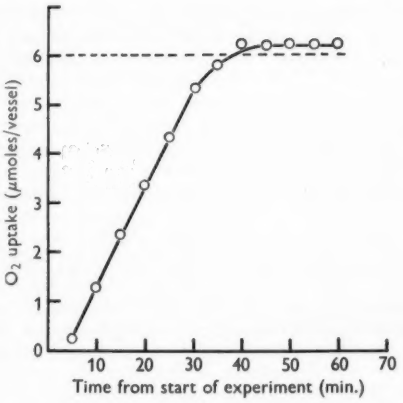

Fig. 1. Oxygen uptake by *Nitrobacter* in the presence of nitrite. The Warburg vessel contained 2·5 ml. of *Nitrobacter* suspension in 0·03 M phosphate buffer, pH 7·8. Sodium nitrite solution (0·5 ml.) was added from the side arm after temperature equilibration to give an initial $NaNO_2$ concentration of 4×10^{-3} M. The dotted horizontal line represents the O_2 uptake corresponding to complete oxidation of the added NO_2^- to NO_3^-.

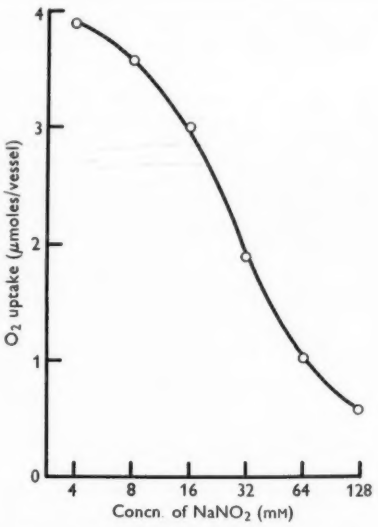

Fig. 2. Effect of nitrite concentration on the rate of O_2 uptake by *Nitrobacter*. Experimental conditions were the same as those for Fig. 1 except that different amounts of $NaNO_2$ were added as 0·5 ml. of solution from the side arms to give the initial $NaNO_2$ concentrations shown on the abscissa. Oxygen uptakes were measured for each flask 30 min. after addition of the nitrite.

In the presence of chlorate, the rate of nitrite oxidation by *Nitrobacter* suspensions was not constant but gradually decreased as the experiment progressed (Fig. 3). In the experiments of Fig. 3, the chlorate was added to the *Nitrobacter* suspension in the main compartment of the Warburg vessels just before temperature equilibration; the nitrite was tipped in from the side arm about 10 min. later, i.e. just after temperature equilibration. Precisely similar results were, however, obtained either when the addition of nitrite from the side arm was delayed for 1 hr. after the chlorate and *Nitrobacter* had been mixed in the main vessel, or when the chlorate and nitrite were mixed in the side arm and added to the *Nitrobacter* together. Where this latter type of experimental procedure was used, the period for which the nitrite and chlorate remained mixed before addition to the *Nitrobacter* suspension did not appear to have any effect on the results.

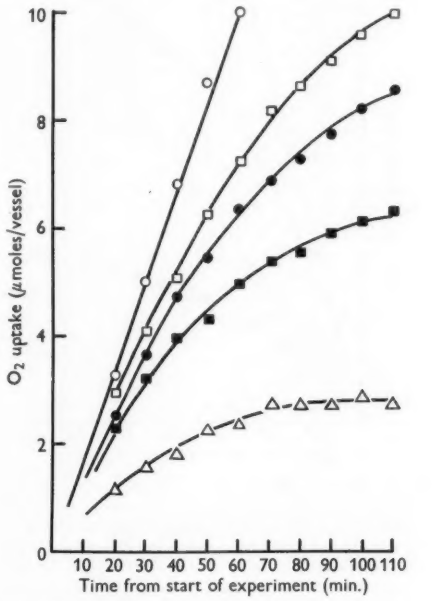

Fig. 3. Effect of $KClO_3$ concentration on the rate of O_2 uptake by *Nitrobacter* in the presence of nitrite. Experimental conditions were similar to those for Fig. 1. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. The $KClO_3$ was added to the cell suspension in the main vessel before temperature equilibration; the concentrations of $KClO_3$ in the main vessels were: $1·7 \times 10^{-2}$ M (△); $8·3 \times 10^{-3}$ M (■); $5·6 \times 10^{-3}$ M (●); $4·2 \times 10^{-3}$ M (□); nil (○).

The inhibition of nitrite oxidation caused by 8×10^{-3} M-$KClO_3$ remained virtually unchanged when the substrate concentration was raised from $2{\cdot}5 \times 10^{-3}$ M-$NaNO_2$ to 5×10^{-3} M-$NaNO_2$. Attempts to reverse the inhibition by nitrate were unsuccessful; concentrations of up to 5×10^{-2} M-$NaNO_3$ were tested. The effect of cell density on the inhibition of nitrite oxidation by chlorate is shown in Fig. 4.

When *Nitrobacter* suspensions were pre-incubated with chlorate alone (concentrations of up to 2×10^{-2} M-$KClO_3$ were tested) or with nitrite alone (up to 0·1 M-$NaNO_2$) for 1 hr., washed, and then tested in the Warburg apparatus, the ability of the suspensions to oxidize nitrite was unchanged. Cells pre-incubated with chlorate and nitrite together oxidized nitrite, after washing, at a constant rate. The rate lessened as the period of pre-incubation was increased (Table 1).

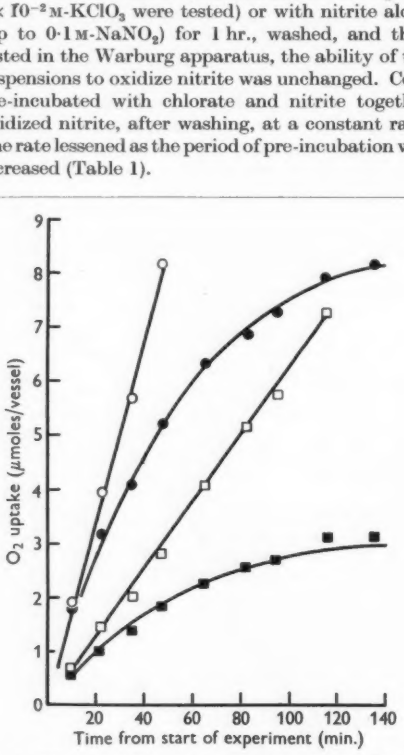

Fig. 4. Effect of cell density on the rate of O_2 uptake by *Nitrobacter* in the presence of nitrite and in the presence and absence of chlorate. Experimental conditions were similar to those for Fig. 1. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. Points ● and ■ indicate that 8×10^{-3} M-$KClO_3$ was present in main vessel, points ○ and □ indicate the absence of chlorate. The cell density of *Nitrobacter* used to determine points ○ and ● was three times that used to determine points □ and ■. The curves through points ● and ■ are not drawn as best fits to the points but are calculated from equation (*c*) (see Discussion). For the upper curve, $KQ_0 = 0{\cdot}168$ μmole of O_2/min., for the lower curve, $KQ_0 = 0{\cdot}062$ μmole of O_2/min.; these values are the slopes of the lines through points ○ and □. For both curves, $kZ = 0{\cdot}019$; this value was obtained by inspection.

Fluoroacetate, nitrourea and various ions of general formula XO_3^- (other than ClO_3^- ions) were tested for their effect on nitrite oxidation by *Nitrobacter*. None proved as effective an inhibitor as chlorate, although the inhibition caused by nitrourea could be markedly increased, either by allowing freshly prepared nitrourea to stand in solution at room temperature for a period, or by treating the solution with alkali (Table 2). Chlorate,

Table 1. *Effect of pre-incubation of* Nitrobacter *cells with nitrite and chlorate on nitrite-oxidizing activity*

Cell suspensions were pre-incubated for various times with 10^{-2} M-$NaNO_2$ and 7×10^{-3} M-$KClO_3$ as shown, washed, and tested for nitrite-oxidizing activity under the experimental conditions of Fig. 1. Sodium nitrite was added from the side arm to give an initial concn. of 10^{-2} M.

Duration of pre-incubation (min.)	Rate of O_2 uptake of the *Nitrobacter* suspension after pre-incubation (μmole of O_2/min.)
0 (control)	0·077
5	0·065
15	0·056
30	0·043
60	0·036
90	0·030

Table 2. *Effect of various compounds on nitrite oxidation by* Nitrobacter

Oxygen uptakes were measured under the experimental conditions of Fig. 3. A solution of the compound to be tested was added to the *Nitrobacter* suspension in the main vessel before temperature equilibration; no compound was added to the control vessels. Sodium nitrite was added from the side arm to give an initial concn. of 8×10^{-3} M in all vessels. Uptakes of oxygen were measured 1 hr. after addition of nitrite.

Compound tested	Concn. of compound (mM)	Percentage inhibition of O_2 uptake
ClO_3^-	10	91
	2	80
BrO_3^-	10	15
	2	8
IO_3^-	10	6
	2	1
PO_3^-	10	2
	2	0
Fluoroacetate	10	32
	2	21
	1	16
Nitrourea, fresh	2	15
Nitrourea, stored 1 day at 15°	2	18
Nitrourea, stored 3 days at 15°	2	41
Nitrourea, alkali-treated*	2	68

* Treated with 10^{-2} M-NaOH for 1 hr. at 15°. Solution neutralized with HCl before testing.

bromate, iodate and fluoroacetate were also tested for their effect on freshly inoculated cultures of *Nitrobacter*. In these cultures, where the *Nitrobacter* population was small, all the compounds completely inhibited nitrite oxidation and therefore, presumably, growth, for a period of at least 9 days at concentrations of 4×10^{-4} M.

Cyanate proved to be a strong inhibitor of nitrite oxidation by *Nitrobacter*. Unlike the inhibition caused by chlorate, nitrite oxidation proceeded at a constant but diminished rate in the presence of cyanate. The degree of inhibition was directly related to the logarithm of the cyanate concentration (Fig. 5). The extent to which the nitrite-oxidizing activities of *Nitrobacter* suspensions were affected by pre-incubation with various mixtures of chlorate, cyanate and nitrite are shown in Table 3.

Chlorite proved to be an inhibitor of nitrite oxidation with an action somewhat between that of chlorate and that of cyanate; there was some increase in the degree of inhibition as the experiment progressed, but the increase was rather less obvious than it was with chlorate (Fig. 6).

Washed, aerated suspensions of *Nitrobacter* showed only very faint absorption bands when examined in a Hartridge reversion spectroscope. When nitrite was added to the suspensions to give a concentration of 10^{-2} M-$NaNO_2$, however, three absorption bands immediately appeared. The sharpest and darkest was a band at 551 mμ.; this was accompanied by a fainter, but almost equally sharp, band at 589 mμ. and a diffuse band at 520–525 mμ. Similar bands could be produced by the addition of dithionite instead of nitrite (Fig. 7). This fact, together with the wavelengths of the bands, has led us to refer to these bands as

Table 3. *Effect of pre-incubation of* Nitrobacter *cells with nitrite, cyanate, and chlorate, on nitrite-oxidizing activity*

Cell suspensions were pre-incubated for 30 min. with 10^{-2} M-$NaNO_2$, 10^{-2} M-$KClO_3$ and 10^{-2} M-NaOCN where indicated (+), washed, and tested for nitrite-oxidizing activity under the experimental conditions of Fig. 1. Sodium nitrite was added from the side arm to give an initial concn. of 8×10^{-3} M.

Substances present during pre-incubation of the *Nitrobacter* suspension			Rate of O_2 uptake of the *Nitrobacter* suspension after pre-incubation
NaOCN	$NaNO_2$	$KClO_3$	(μmole of O_2/min.)
−	−	−	0·29
−	+	−	0·32
+	−	−	0·30
−	−	+	0·29
+	+	−	0·31
−	+	+	0·17
+	−	+	0·30
+	+	+	0·28

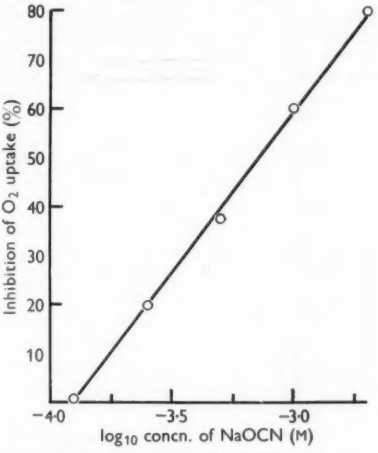

Fig. 5. Effect of cyanate on nitrite oxidation by *Nitrobacter*. Experimental conditions were similar to those for Fig. 1. Sodium cyanate was added at various concentrations to the *Nitrobacter* suspension in the main vessel. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. In the presence and absence of NaOCN, O_2 uptake was linear with time.

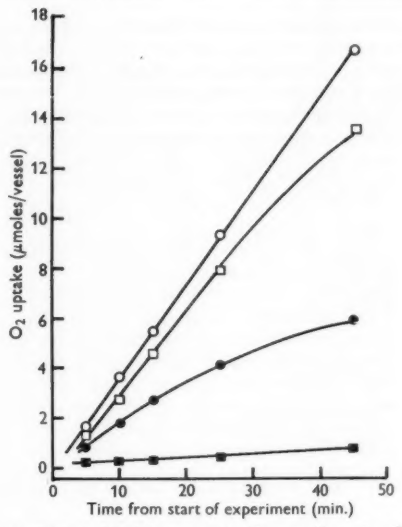

Fig. 6. Effect of $KClO_3$ concentration on the rate of O_2 uptake by *Nitrobacter* in the presence of nitrite. Experimental conditions were similar to those for Fig. 1. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. The concentrations of $KClO_3$ in the main vessels were: 3×10^{-3} M (■); 10^{-3} M (●); 3×10^{-4} M (□); nil (○).

'cytochrome bands'. Suspensions of the heterotrophic contaminant of the *Nitrobacter* cultures (see Materials and Methods) showed no such bands on treatment with nitrite. *Nitrobacter* suspensions treated with 10^{-2}M-$KClO_3$, or with 10^{-2}M-NaOCN followed by 10^{-2}M-$NaNO_2$, showed no cytochrome bands, but the bands could nevertheless be generated by nitrite or dithionite after such suspensions had been washed with water at the centrifuge. *Nitrobacter* suspensions treated with 10^{-2}M-$NaNO_2$ plus 10^{-2}M-$KClO_3$ immediately showed the cytochrome bands, but the bands (particularly that at 551 mμ.) gradually faded if the cells were allowed to remain in contact with the nitrite and chlorate. *Nitrobacter* suspensions whose bands had been allowed to fade in this way failed, after water washing and treatment with nitrite or dithionite, to show cytochrome bands of intensity equal to those shown by a similar suspension not incubated with chlorate and nitrite. The diminution of intensity of the 551 mμ. band, brought about by incubation of the cells with chlorate and nitrite, ran parallel with diminution of nitrite-oxidizing activity (Fig. 8). Chlorite added to a *Nitrobacter* suspension produced an immediate disappearance of the cytochrome bands; the bands could not be regenerated in cells treated with chlorite by water washing at the centrifuge followed by treatment with nitrite or dithionite.

In attempts to find some electron acceptor (other than atmospheric oxygen) for nitrite oxidation by *Nitrobacter*, cell suspensions, together with nitrite in 0·03 M phosphate buffer, pH 7·8, were incubated anaerobically for 1 hr. in the presence of triphenyltetrazolium chloride, methylene blue, methylene

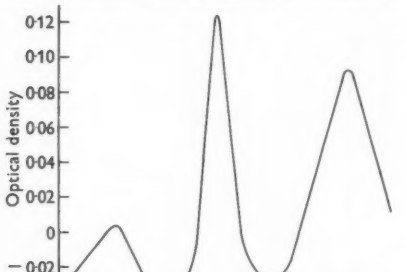

Fig. 7. Absorption spectrum of *Nitrobacter* cells in the presence of dithionite. Optical densities recorded are difference values recorded against the 'blank' values given by a thoroughly aerated *Nitrobacter* suspension containing no dithionite. Similar spectra were given by *Nitrobacter* suspensions made 10^{-2}M with respect to $NaNO_2$.

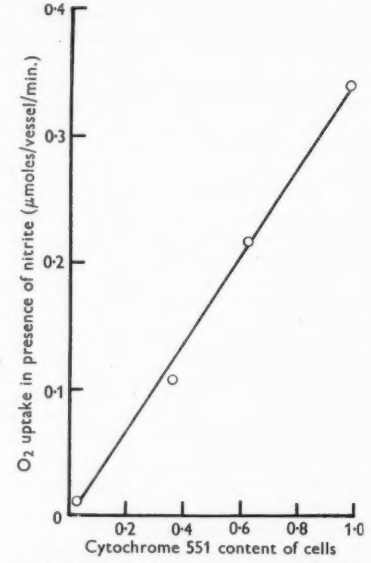

Fig. 8. Relation between cytochrome 551 content and nitrite-oxidizing ability of *Nitrobacter*. The cytochrome 551 initially present in the *Nitrobacter* cells was partially destroyed by pre-incubation of the cells with 10^{-2}M-$KClO_3$ and 10^{-2}M-$NaNO_2$. The amount of cytochrome 551 remaining in the cells after pre-incubation was determined as the ratio 'optical density at 551 mμ.: optical density at 589 mμ.' (see Materials and Methods) after treatment with dithionite. The nitrite-oxidizing ability of the pre-incubated cells was determined under experimental conditions similar to those for Fig. 1.

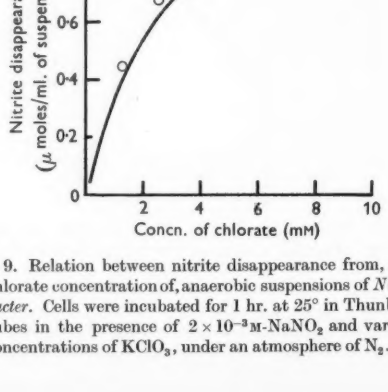

Fig. 9. Relation between nitrite disappearance from, and chlorate concentration of, anaerobic suspensions of *Nitrobacter*. Cells were incubated for 1 hr. at 25° in Thunberg tubes in the presence of 2×10^{-3}M-$NaNO_2$ and various concentrations of $KClO_3$, under an atmosphere of N_2.

blue plus adenosine triphosphate, mammalian cytochrome *c*, water extract of baker's yeast, or chlorate. The nitrite concentration remained unchanged in all preparations except that containing chlorate; here there was a slight fall in nitrite concentration. This fall did not occur when boiled *Nitrobacter* suspensions were used. Further experiments showed that the fall in nitrite concentration was related to the concentration of chlorate used (Fig. 9).

DISCUSSION

The work here described is, to the best of our knowledge, the first detailed biochemical investigation of the mechanism whereby *Nitrobacter* oxidizes nitrite to nitrate. Because there is as yet no method of growing *Nitrobacter* in reasonable quantity, our results are to some extent fragmentary. Nevertheless, in view of our findings we feel justified in suggesting that the following reactions occur during nitrite oxidation by *Nitrobacter*:

*(i) $Fe^{3+}_{cyt.\ 551} + NO_2^- \rightarrow Fe^{2+}_{cyt.\ 551} + NO_2$.

(ii) $Fe^{2+}_{cyt.\ 551} + NO_2 + \frac{1}{2}O_2 \rightarrow Fe^{3+}_{cyt.\ 551} + NO_3^-$.

(iii) $Fe^{2+}_{cyt.\ 551} + NO_2 + ClO_3^- \rightarrow Fe^{3+}_{cyt.\ 551} + NO_3^- + ClO_2^-$.

(iv) $Fe^{3+}_{cyt.\ 551} + ClO_2^- \rightarrow$ inactivated cytochrome 551.

* Reaction (i) is reversibly inhibited by cyanate.

This scheme is intended as an outline, not as a precise formulation, of the processes taking place in *Nitrobacter*. The symbol NO_2 does not necessarily represent the nitrite radical, it merely represents some compound, free or bound to a carrier, at the oxidation level of the nitrite radical. Although the reactions are depicted as taking place directly, this does not preclude the possibility that one or more of them may involve carrier systems. Our evidence for the scheme is as follows:

Reaction (*i*). $Fe^{2+}_{cyt.\ 551}$ is produced when nitrite is added to *Nitrobacter* suspensions and the rate of nitrite oxidation depends on the amount of cytochrome 551 in the cell (Fig. 8). Addition of cyanate to *Nitrobacter* suspensions containing nitrite inhibits nitrite oxidation (Fig. 5) and, if present in sufficiently high concentration (see Results), prevents the appearance of the band of $Fe^{2+}_{cyt.\ 551}$. The effect of cyanate is, however, reversible by water washing. These results suggest that cyanate has no effect on the cytochrome 551 itself, but prevents the cytochrome 551 from accepting electrons from nitrite.

Reaction (*ii*). This reaction (i.e. the reoxidation of $Fe^{2+}_{cyt.\ 551}$ by atmospheric oxygen) must take place, although it may not take place directly; it may involve intermediate carriers. In the absence of nitrite, the spectrum of a *Nitrobacter* suspension shows no reduced cytochrome bands; these bands appear immediately when nitrite is added to the suspension and disappear when all the nitrite has been oxidized. This suggests that reaction (ii) rather than reaction (i) limits the rate of nitrite oxidation in *Nitrobacter*. That reaction (ii) is slower than reaction (i) also harmonizes with the fact that, although the nitrite concentration in a *Nitrobacter* suspension steadily falls as nitrite is oxidized to nitrate, the rate of nitrite oxidation by such suspension remains fairly constant until almost all the nitrite has been oxidized (Fig. 1).

Reaction (*iii*). That chlorate can act as an oxidizing agent in nitrite oxidation by *Nitrobacter* follows from the results of Fig. 9, which show that nitrite disappears from *Nitrobacter* suspensions under anaerobic conditions in the presence of chlorate. Owing to the fact that nitrate cannot be estimated satisfactorily in the presence of relatively large amounts of chlorate, we were not able to show that the nitrite disappearing was oxidized to nitrate, but since chlorate cannot easily be visualized as a reducing agent, it seems almost certain that the nitrite was indeed so oxidized. Indirect evidence for the occurrence of reaction (iii) is provided by the fact that chlorate inhibition of nitrite oxidation only becomes apparent when nitrite oxidation is actually proceeding, during which process the inhibition becomes progressively greater (Table 1; Figs. 3, 4).

Reaction (*iv*). The rapid destruction of cytochrome 551 by chlorite has been observed spectroscopically; it seems probable that this destruction is similar to the destruction of metmyoglobin observed by George & Irvine (1954). Presumably because it destroys cytochrome 551, chlorite proved to be a powerful inhibitor of nitrite oxidation by *Nitrobacter* (Fig. 6).

Since all our results accord with the operation of reactions (i)–(iv), it seems probable that these reactions describe reasonably well the mechanism of nitrite oxidation in *Nitrobacter*. There are, however, some ancillary points worth consideration.

Simpson (1955) has shown that the kinetics of the inhibition of nitrite oxidation by chlorate may be adequately explained on the basis of two postulates:

(*a*) The rate of nitrite oxidation in a *Nitrobacter* suspension is proportional to the amount (Q_t) of cytochrome 551 in the cells at time 't' (see Fig. 8), i.e.

$$-\frac{d(NO_2)}{dt} = KQ_t,$$

where K is a constant and (NO_2) is the amount of nitrite.

(*b*) The rate of destruction of cytochrome 551 in a *Nitrobacter* suspension containing chlorate at

concentration Z is proportional to Z and to Q_t (see reactions (iii) and (iv)), i.e.

$$-\frac{dQ}{dt} = kZQ_t,$$

where k is a constant. Integration and combination of these two expressions yields the equation:

$$(c) \qquad -(NO_2)_t = \frac{KQ_0}{kZ}(1 - e^{-kZt}),$$

where $-(NO_2)_t$ = amount of nitrite oxidized after time 't'; KQ_0 = initial rate of nitrite oxidation, i.e. = rate of nitrite oxidation in the absence of chlorate. The fit of equation (c) to the experimental points is shown in Fig. 4. When 't' is very large, equation (c) approximates to

$$(d) \qquad -(NO_2)_t = \frac{KQ_0}{kZ}.$$

This equation (d) predicts that, for a given concentration of chlorate, the amount of nitrite oxidized to nitrate before oxidation becomes vanishingly slow depends on the value of Q_0; that is, it depends on the cell density of the *Nitrobacter* suspension used. This prediction not only agrees with the results of Fig. 4, it also offers a possible explanation of the results of Lees & Quastel (1945), which showed that chlorate at concentrations of about 10^{-5} M virtually prevented nitrite oxidation in soils with a low *Nitrobacter* population, but had little effect on nitrite oxidation in soils with a high *Nitrobacter* population. Lees & Quastel also found that the inhibitory action of 10^{-5} M chlorate could be abolished by nitrate at a concentration of about 10^{-2} M. These results are explicable on the basis of reaction (iii). Since nitrate is an end product of the reaction, the forward reaction should be inhibited by nitrate. Thus in the presence of nitrate, chlorite formation should be minimized and destruction of cytochrome 551 should be reduced. Our failure to diminish the inhibitory effect of chlorate by addition of nitrate may be explained by the fact that, at the high cell densities of *Nitrobacter* used in the present experiments, no possible concentration of nitrate could appreciably reverse reaction (iii). Lees & Quastel (1945) reversed chlorate inhibition of nitrite oxidation by a molar ratio of chlorate : nitrate of approximately $1 : 10^3$. Since the chlorate concentrations in our experiments have been of the order of 10^{-2} M, no reversal by nitrate could have been expected much below a concentration of 10 M nitrate. At this concentration of nitrate *Nitrobacter* could scarcely be expected to operate at all. Considerations of cell density probably explain also why iodate and bromate, although they only weakly inhibited nitrite oxidation in experimental *Nitrobacter* suspensions (Table 2), strongly inhibited oxidation in freshly inoculated cultures (see Results). Quastel & Scholefield (1951) found that even in soils with a high *Nitrobacter* population, potassium chlorate at a concentration of 6×10^{-3} M inhibited nitrite oxidation. This agrees with the results presented here. The failure of fluoroacetate to inhibit nitrite oxidation by *Nitrobacter* (Table 2), although it inhibited growth of *Nitrobacter*, is probably to be explained on the grounds that it interfered with the mechanisms of the organisms which assimilate carbon dioxide rather than with their nitrite-oxidizing mechanisms.

Nitrourea in aqueous solution decomposes to cyanate, especially under alkaline conditions (Davis & Blanchard, 1929). This probably explains why solutions of nitrourea inhibited nitrite oxidation more strongly after standing or alkali treatment (Table 2). The reported inhibition by nitrourea of nitrite oxidation by *Nitrobacter* in soil (Quastel & Scholefield, 1951) may likewise be due to the transformation of nitrourea into cyanate during percolation through the soil.

We are now trying to grow *Nitrobacter* on a scale sufficiently large to enable us to separate the cytochrome systems. By this means we hope to study more closely the details of the mechanisms outlined in reactions (i)–(iv).

SUMMARY

1. The mechanism of nitrite oxidation has been studied in the autotrophic bacterium *Nitrobacter*.

2. Cyanate inhibited nitrite oxidation in *Nitrobacter*: the effect was reversible by washing the cells with water.

3. Chlorate did not inhibit nitrite oxidation directly but was converted, during the course of nitrite oxidation, into some compound (possibly chlorite) that did inhibit nitrite oxidation. This inhibition could not be reversed by washing with water.

4. The results of these studies with cyanate and chlorate, together with results from spectroscopic examination of *Nitrobacter* cells under various conditions, suggest that a cytochrome with an absorption maximum at 551 mμ. in the reduced state is intimately concerned with nitrite oxidation by *Nitrobacter*.

We wish to thank Professor W. O. Kermack, F.R.S., for his advice and encouragement throughout this work. One of us (J.R.S.) also wishes to acknowledge the receipt of a grant from the Agricultural Research Council.

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The Annelid Phosphokinases

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Roche and his co-workers (Thoai, Roche, Robin & Thiem, 1953; Thoai & Robin, 1954) have described the isolation of taurocyamine and glycocyamine from a number of annelid worms and this work has been confirmed by Ackermann (1955*a*, *b*). It was suggested that these bases when phosphorylated on the terminal amino group functioned as 'phosphagens'. Thoai *et al.* (1953) and Hobson & Rees (1955) showed that these bases and creatine were present as the phosphates in annelid muscle. Further support for this concept is given by the evidence presented in this paper, that the annelids contain phosphokinases capable of phosphorylating taurocyamine, glycocyamine and creatine at the expense of adenosine triphosphate (ATP); the reactions can be expressed as a generalized version of the Lohmann reaction: guanidine base + ATP⇌ phosphagen + ADP. A preliminary report of this work was presented by Hobson (1955).

METHODS

Extraction of enzymes. The animal was cooled in sea water until it was torpid; the gut was removed where this was practicable. To the weighed muscle of the body wall was added 2 vol. of ice-cold distilled water and the tissue was ground in a cold mortar with a little acid-washed sand. The extract was allowed to stand at 0° for 10 min. and then 5 vol. of cold acetone was added with stirring. The extract was filtered and the acetone-dried powder placed in a vacuum desiccator over anhydrous $CaCl_2$. The powder proved stable for several months if stored at 2°.

For the transphosphorylation experiments 2 g. of the acetone powder was ground with 10 ml. of distilled water and the extract dialysed against water for 15 hr. at 0°. The insoluble material was removed by centrifuging and the clear supernatant used for the experiments.

Transphosphorylation system. This was based on the system described by Lardy, Kuby & Noda (1954*a*, *b*) for their studies on creatine phosphokinase. The final concentrations present were: guanidine base, 0·024M; ATP, 0·005M; $MgSO_4$, 0·0003M; glycine buffer, 0·1M (pH 9·0); 0·4 ml. of enzyme soln. was added. The final vol. was 3 ml. and incubation was for 15 min. at 40°. This system was found to give maximum phosphorylation, the addition of all components being necessary for phosphorylation of the base. The reaction was completed in about 10 min. and there was no evidence of inactivation of the enzyme at this temperature nor of any breakdown of the phosphagens before analysis.

Materials. Creatine, taurocyamine and glycocyamine were commercial preparations (L. Light and Co. Ltd.). ATP was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). Creatine phosphate was prepared by the method of Ennor & Stocken (1948).

Determinations. The quantity of phosphagens was determined after transphosphorylation experiments as follows. The reaction was stopped by the addition of 2 ml. of ice-cold 20% (w/v) trichloroacetic acid to the incubation mixture. The mixture was cooled for 5 min. in crushed ice and then centrifuged at 600 *g* for 5 min. at 0°. The clear supernatant was neutralized (pink to phenolphthalein) and the $Ca(OH)_2$–$CaCl_2$ reagent of Fiske & Subbarow (1929) was added to precipitate the inorganic and nucleotide phosphates. After standing for 10 min. in ice the tubes were centrifuged. The phosphagens were estimated in the supernatants by the method described by Baldwin & Yudkin (1950).

The solvents used in chromatographic analysis of the guanidine phosphates and guanidine bases were as described by Hobson & Rees (1955). The chromatograms were run at 26·5° and not, as reported earlier by us, at 22°.

RESULTS

Enzyme extracts were prepared from a number of errant and sedentary polychaete worms and portions (0·4 ml.) were incubated in the standard medium with each of the four substrates, taurocyamine, glycocyamine, arginine and creatine. The

results (Table 1) of a series of experiments show that one or other guanidine phosphate was formed in each case.

To characterize the product of the reaction, the phosphagens were separated and chromatographed. They were freed from the substrates after the addition of trichloroacetic acid and calcium reagent by precipitation at 0° with 3 vol. of ice-cold acetone, and then dissolved in the minimum quantity of water. This procedure was repeated twice. The solutions were either chromatographed directly, phosphagens extracted directly from the muscle of the animal from which the enzyme had been prepared being used as markers, or alternatively phosphagens were hydrolysed with N-HCl at 100° for 5 min. and the free bases chromatographed against markers of known identity. In all cases, as had been previously reported by Hobson & Rees (1955), the R_F value of the base liberated by hydrolysis of the phosphagens was the same as the R_F value of the base which had been added to the incubation medium. The R_F values of the synthesized and extracted phosphagens (Table 2) also agreed. For comparison the R_F value of chemically synthesized creatine phosphate is included.

DISCUSSION

It is assumed that where a base is phosphorylated to any appreciable extent this can be taken as evidence for the view that the compound produced would act as a phosphagen *in vivo* fulfilling the same functions as the well-known phosphagens, arginine and creatine phosphates, in muscular activity. Although the enzyme extracts from the different species were not found to be specific for a single base, the work described here supported the earlier evidence for the existence of new phosphagens (Thoai *et al.* 1953;

Table 1. *Enzymic synthesis of the guanidine phosphates*

Medium for transphosphorylation as described in the Methods section.

	Guanidine phosphate formed (μmoles)			
Source of enzyme preparation	Taurocyamine phosphate	Glycocyamine phosphate	Arginine phosphate	Creatine phosphate
Arenicola marina (body wall)	5·0	1·25	0	0
Nereis diversicolor	0·4	6·5	0	0
N. fucata	0	5·8	0	2·0
Hermione hystrix	0	0	0	4·5
Aphrodite aculeata	0	0	0	1·25
Myxicola infundibulum	1·5	0	0	0
Nephthys cacea	0	1·7	0	1·7

Table 2. *Chromatographic analysis of the guanidine phosphates*

Method of preparation or isolation of the phosphagens is described in the text. TP, GP and CP represent taurocyamine, glycocyamine and creatine phosphate respectively.

		Solvent systems	
		Propan-1-ol–NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.)	*iso*Pentanol–pyridine–water (4:8:7, by vol.)
Source of phosphagen	Nature of phosphagen	R_F values	
Arenicola marina body wall	TP	0·24	0·19
Synthesized with *A. marina* enzyme	TP	0·23	0·18
Nereis diversicolor	GP	0·20	0·08
Synthesized with *N. diversicolor* enzyme	GP	0·19	0·09
Glycera gigantea body wall	TP	0·24	—
	CP	0·29	—
Synthesized with *G. gigantea* enzyme	TP	0·24	—
	CP	0·29	—
Hermione hystrix	CP	0·30	—
Synthesized with *H. hystrix* enzyme	CP	0·31	—
Chemically synthesized	CP	0·30	—

Hobson & Rees, 1955) and served to identify them by synthetic as opposed to degradative means. Throughout the species investigated the phosphokinases predicted by the previous results were all proved to be present and capable of phosphorylating the expected base.

As was reported for some of the echinoderms (Needham & Baldwin, 1937), several annelids, e.g. *Glycera gigantea*, *Nereis diversicolor* and *Myxicola infundibulum*, contain a mixture of phosphagens and show that the enzyme systems for the synthesis and utilization of phosphagens in living muscle are completely duplicated.

The experimental results again emphasize that creatine phosphate, acting as a phosphagen, is not confined to the echinoderms, protochordates and vertebrates, but is also present, often in considerable quantities, in the annelids. At the present time the distribution of creatine phosphate appears quite arbitrary. This fact lessens the value of the identification of the phosphagens in evolutionary studies in the animal kingdom.

SUMMARY

1. A method of extraction of guanidine phosphokinases from annelid muscle is described.

2. Transphosphorylation has been demonstrated and the synthesized guanidine phosphates have been identified.

3. It is concluded that taurocyamine and glycocyamine phosphate as well as creatine phosphate can act as phosphagens in certain of the annelids.

We would like to express our appreciation to the staff of the Marine Biological Laboratory, Plymouth, who provided much living material, and to the administrators of the Field Fund of University College, London, for a grant to cover part of this work. Also our thanks are due to Professor E. Baldwin for his advice and encouragement in this work.

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Protein Synthesis in Guinea-pig Liver

INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEINS OF THE MICROSOME FRACTION *IN VIVO*

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When a labelled amino acid is injected intravenously into normal animals the liver protein becomes rapidly labelled. Fractionation of the liver subcellular components by differential centrifuging has shown that there is a high rate of incorporation of amino acid into the proteins of the microsome fraction (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950; Keller, 1951). It has been shown, also, that most of the cytoplasmic ribonucleic acid is concentrated in the microsome fraction and that, at least with pancreas, digestion of the microsomes by ribonuclease releases a protein with a particularly high turnover rate (Allfrey, Daly & Mirsky, 1953).

The present investigation was designed to gain additional information concerning the role of the microsome fraction in protein synthesis. It was found, following a suggestion by Dallam (1955), that further fractionation of the microsome material of guinea-pig liver could be achieved by extraction with solutions of varying ionic strength and pH. This method was used to follow the rate of incorporation of radioactive amino acids into different microsome proteins after injection of a mixture of [^{14}C]amino acids into normal guinea pigs. Our results indicate that the microsome fraction contains a complex mixture of proteins with widely differing turnover rates, the protein fraction with the highest turnover

rate being associated with ribonucleic acid. This connexion between rapid protein turnover and attachment to ribonucleic acid may be significant, since two other laboratories have obtained rather similar results using different methods of fractionation (Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955). A preliminary account of the present investigation has been published (Simkin, 1955).

EXPERIMENTAL

Chemical analyses

Total nitrogen. Estimations were by a micro-Kjeldahl procedure.

Protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), whole microsome protein being used as standard (the proteins of all fractions gave the same optical density value per unit weight with the reagent).

Ribonucleic acid. The orcinol method of Mejbaum (1939) was used to estimate ribonucleic acid (RNA). A sample of guinea-pig liver cytoplasmic RNA, isolated by the method of Davidson, Frazer & Hutchison (1951), was used as standard.

Deoxyribonucleic acid. The diphenylamine reaction as described by Burton (1955) was used for estimation of deoxyribonucleic acid (DNA).

Phosphorus. The method of Allen (1940) was used, but with the total volume scaled down to 5 ml.

MATERIALS

Animals. Guinea pigs from the Institute stock were employed (750–900 g., about 7 months old). The animals were starved overnight before the experiment. All injections were intravenous.

Radioactive amino acids. A mixture of uniformly labelled L-[^{14}C]amino acids was prepared by acid hydrolysis of [^{14}C]chlorella protein; the latter was supplied by the Radiochemical Centre, Amersham, Bucks. Each animal received about 35 μc/kg. (7·5 μmoles of N/kg.).

Preparation of homogenates

Removal of liver. For the 2 min. experiment, the method was as follows: the dose was injected over a period of 5 sec. and the animal killed by a blow on the head 120 sec. later. The body was plunged into a bath of ice-cold physiological saline, the abdominal wall was opened immediately and the liver removed. The whole operation required 150 sec. from the completion of the injection. For experiments at longer time intervals, the cold bath was not used but the liver was quickly transferred to ice-cold saline. All homogenates and subcellular fractions were maintained at 0–5° throughout.

Method of homogenizing. The liver was minced and freed from connective tissue by forcing through a metal screen with holes of 1 mm. diameter. The liver mince (approximately 25 g.) was mixed with an equal weight of 0·25 M sucrose, and homogenized in a Potter–Elvehjem type homogenizer, with a polytetrafluoroethylene pestle (referred to below as a Teflon homogenizer). The difference between the internal diameter of the tube and the diameter of the pestle was 0·25 mm. The pestle was driven at 2000 rev./min. and the liver was homogenized for 45 sec.; about 15–20 up-and-down strokes of the pestle were completed during this period. Twice the original volume of 0·25 M sucrose was then added to the homogenate and a sample (2 ml.) was taken for determination of the radioactivity of the whole liver protein.

Preparation of the microsome fraction and the cell sap

Whole cells, nuclei and mitochondria were removed from the diluted sucrose homogenate by centrifuging in a refrigerated, angle-head centrifuge (centrifuge *B*). The homogenate was spun for 10 min. at 7000 rev./min. This corresponded to 5000 g ($g_{max.}$) at the maximum radius (r = 9·5 cm.) and 4000 g ($g_{av.}$) at the average radius. The supernatant was removed by suction, the small amount of supernatant immediately above the sediment being rejected. The supernatant was then recentrifuged for 15 min. at 8200 rev./min. ($g_{max.}$ = 7000; $g_{av.}$ = 5500). The supernatant was collected, a small amount of supernatant overlying the sediment being discarded. At this stage only a few particles of the size of mitochondria could be found in the supernatant (ultraviolet phase-contrast microscopy). The supernatant was diluted to 100 ml. with 0·25 M sucrose (this involved diluting with slightly less than an equal volume of sucrose). The diluted supernatant was then centrifuged in a Spinco Model E ultracentrifuge, with preparative rotor A, for 45 min. at 42040 rev./min. ($g_{av.}$ = 103000). The supernatant was removed by suction, that part of the supernatant immediately above the sediment of microsome material being rejected. The supernatant obtained at this stage is referred to hereafter as the cell-sap fraction.

Preparation of washed microsomes. The microsome pellet separated from the cell sap was washed twice with 2–3 ml. of 0·25 M sucrose, by layering sucrose over the pellet, inverting the tube and allowing to drain. The microsome fraction was then washed twice with glycerol, as suggested by Dallam (1955). Aqueous glycerol was used at this stage to obviate the need for ultracentrifuging. Often a trace of brown-coloured material was seen underlying the pink microsome material (this brown material is presumably of mitochondrial origin; cf. Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). It was possible to reject this material while removing the microsome sediment completely from the centrifuge tube. The microsome fraction was dispersed in about 10 ml. of 38% (v/v) glycerol (A.R.) by a Teflon homogenizer driven at 1000–1500 rev./min. The suspension was diluted to 50 ml. by the addition of 38% glycerol, and then centrifuged in centrifuge *B* for 35–40 min. at 17000 rev./min. ($g_{max.}$ = 30000, $g_{av.}$ = 25 000; deceleration from such speeds took about 10 min.). Sometimes a small amount of material (estimated at about 5% or less) failed to sediment. The supernatant, containing the unsedimented material, was discarded. The sediment was washed again in a similar manner. Complete sedimentation was always achieved in the second washing. The supernatant was discarded and the centrifuge tubes containing the sediment were drained. This sediment will be referred to as the washed microsome fraction. A sample of this material was taken for determination of the radioactivity (or analysis) of the whole microsome fraction.

Fractionation of washed microsomes

Immediately before the extraction procedure was begun, glycerol was removed from the surface of the washed microsome sediment by adding about 5 ml. of 0·14 M-NaCl to the centrifuge tube, and pouring off the solution, the whole operation being carried out as rapidly as possible.

Each extraction was carried out by suspending the material in the appropriate solution, a Teflon homogenizer driven at about 1500 rev./min. being used. The suspension was allowed to stand for 15–20 min. and the insoluble material was then removed by centrifuging for 15 min. in centrifuge *B* at 17 000 rev./min. The supernatant was collected by suction.

The details of the fractionation scheme are given in Fig. 1. Usually, fractionation was started with washed microsome material containing about 25 mg. of N. This quantity was obtained from about 15 g. of liver.

In some early experiments, fraction *C* was extracted by suspending insoluble material from the previous step in 0·14 M-NaCl (0·5 ml./mg. of N) and adding an equal volume of 0·01 N-NaOH; the extract had the usual composition of fraction *C*.

Isolation of protein for radioactivity measurement

Protein was precipitated from all samples by the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA); the complete homogenate and whole microsome fraction samples were diluted about 5 times before precipitation. The precipitate was centrifuged down, the supernatant discarded, and the precipitate redissolved in warm N-NaOH (3–10 ml. according to the amount of precipitate). A large excess of an unlabelled amino acid mixture was then added in the form of a protein hydrolysate, 2–5 μmoles of hydrolysate N (0·5–1·0 ml.) being added/ml. of NaOH. The protein was then reprecipitated with TCA. The protein precipitate was washed as follows: once with 5% (w/v) TCA at 20°, once with 5% TCA, heating for 15 min. at 90° before centrifuging, once with 5% TCA at 20°, twice with acetone at 20°, twice with 3:1 (v/v) ethanol–ether at 60° and finally twice with ether at 20°. The protein precipitates were then air-dried, usually at 37°.

As the liver from the animal killed at 2 min. was likely to contain much labelled free amino acids, a TCA solution which contained casein hydrolysate (4 μmoles of N/ml.) was used to precipitate protein in the first step. The remainder of the isolation procedure was as described above.

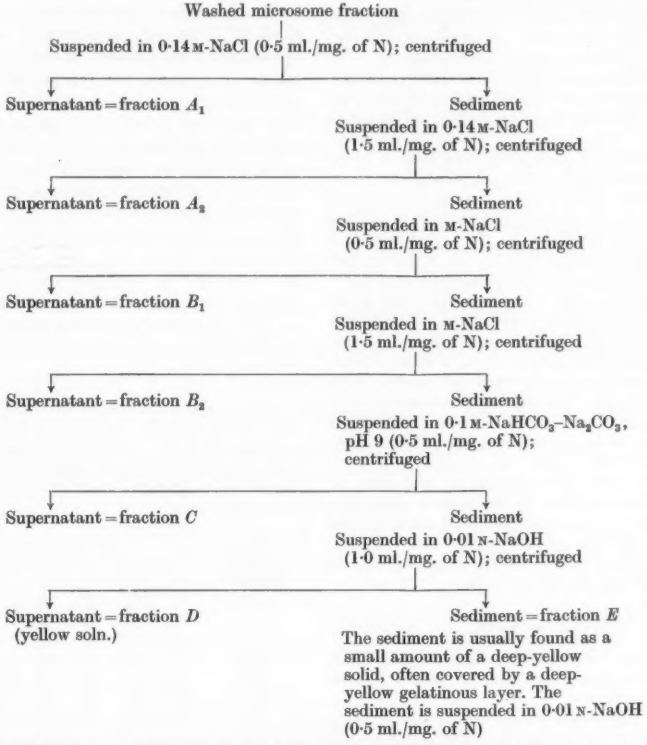
Washed microsome fraction
Suspended in 0·14 M-NaCl (0·5 ml./mg. of N); centrifuged
Supernatant = fraction A_1
Sediment
Suspended in 0·14 M-NaCl (1·5 ml./mg. of N); centrifuged
Supernatant = fraction A_2
Sediment
Suspended in M-NaCl (0·5 ml./mg. of N); centrifuged
Supernatant = fraction B_1
Sediment
Suspended in M-NaCl (1·5 ml./mg. of N); centrifuged
Supernatant = fraction B_2
Sediment
Suspended in 0·1 M-$NaHCO_3$–Na_2CO_3, pH 9 (0·5 ml./mg. of N); centrifuged
Supernatant = fraction *C*
Sediment
Suspended in 0·01 N-NaOH (1·0 ml./mg. of N); centrifuged
Supernatant = fraction *D* (yellow soln.)
Sediment = fraction *E*
The sediment is usually found as a small amount of a deep-yellow solid, often covered by a deep-yellow gelatinous layer. The sediment is suspended in 0·01 N-NaOH (0·5 ml./mg. of N)

Fig. 1. Scheme for fractionation of microsome material. The volumes of extracting solutions quoted are given in terms of ml./mg. of nitrogen of the original washed microsome material taken for extraction.

The protein was not isolated from subfractions A_2 and B_2 owing to the low concentration of material contained in these fractions.

Determinations of radioactivity. The dried protein samples were counted on 0·3 cm.² or 1·0 cm.² polythene planchets at infinite thickness, with an end-window Geiger counter.

Analyses of microsomes and microsome subfractions

The method used for isolation of protein for radioactivity determination was designed to ensure absence of contamination by traces of free radioactive amino acids. For analyses of the various microsome subfractions a somewhat simpler procedure was used.

Protein and lipid-soluble phosphorus. A sample of 1·0–1·5 ml. of the appropriate fraction (2·0–3·0 ml. for fractions A_2 and B_2) or of a 1:5 dilution of the whole microsome fraction suspension was taken, and an equal volume of 10% (w/v) TCA added. The precipitates were washed by the use of a procedure similar to that described above for isolation of protein, except that the stage of re-solution in NaOH and reprecipitation was omitted, and the precipitates were washed twice with cold TCA after extraction with hot TCA; 1·5 ml. of extractant was used at each stage. The acetone, ethanol–ether and ether extracts were pooled, the solvents removed *in vacuo*, and the total phosphorus content of the residues was determined (lipid-soluble P). The precipitate remaining after ether extraction was dissolved in warm N-NaOH, and the protein in solution determined.

Estimation of ribonucleic acid and acid-soluble nucleotide. RNA was separated from other constituents in the following manner. An equal volume of cold M-$HClO_4$ was added to each sample at 0° (volumes as for procedure described immediately above). The precipitate was washed twice with 1·0 ml. of chilled 0·5M-$HClO_4$. The original supernatant and the two washings were pooled, and will be referred to as the acid-soluble nucleotide fraction. Lipid was extracted from the precipitate by three washings with 1·0–1·5 ml. of ethanol–ether–$CHCl_3$ (2:2:1, v/v); the first two extractions were carried out at 20° and the last at 50°. In the first extraction, the solvent mixture was made 0·1 M with respect to potassium acetate. The precipitate was then suspended in 2·0 ml. of M-$HClO_4$ and allowed to stand at room temperature overnight (cf. Ogur & Rosen, 1950). The precipitate was centrifuged down and washed twice with 1·0 ml. of M-$HClO_4$. The digest supernatant and the two $HClO_4$ washings were combined and taken as the RNA digest. The pentose content of both the acid-soluble nucleotide fraction and the RNA digest was determined.

Deoxyribonucleic acid. The separation procedure of Schneider (1945) was applied to the various microsome subfractions and to the washed microsome fraction. For each estimation, the amount of material used was three times that used for RNA estimation. The material extracted by 5% TCA at 90° was assayed for DNA.

Physical methods

Ultraviolet-absorption curves. The ultraviolet-absorption curves of the fractions were determined with a Unicam SP. 500 spectrophotometer.

Electrophoresis and sedimentation studies. Preliminary investigations into the electrophoretic and sedimentation characteristics of fractions A_1, B_1 and C were carried out. Because of the low concentration of material present in these fractions, it was necessary to concentrate each fraction several-fold. The fractions were dialysed against appropriate strength veronal–NaCl buffers and the dialysed materials freeze-dried. The concentrated preparations of fractions A_1 and C were examined in a Perkin–Elmer Tiselius electrophoresis apparatus, with veronal buffer of pH 8·4, $I = 0·1$, and a current of 12 mA. The concentrated preparations of fractions A_1, B_1 and C were analysed in the Spinco Model E ultracentrifuge at 59 780 rev./min. with analytical rotor A and a 12 mm. cell, with veronal buffers of pH 8·4; the ionic strengths were 0·15 for fractions A_1 and C, and 0·3 for fraction B.

RESULTS

Incorporation of [¹⁴C]amino acids into the proteins of liver-cell fractions

Table 1 records the radioactivity of proteins from whole liver, washed microsome fraction and cell-sap fraction obtained from guinea pigs killed at different times after the intravenous injection of a mixture of [¹⁴C]amino acids ([¹⁴C]chlorella-protein hydrolysate). During the first 30 min. there is a rapid increase in the radioactivity of the protein of whole liver; thereafter, the increase in activity is more gradual. There is, however, a very rapid initial uptake into the protein of the microsome fraction, which also lasts for about 30 min., this being followed by a period in which there is only a very slow increase in activity; thus, after 2 min., the microsome protein has about four times the activity of the whole liver protein. The soluble protein of the cell-sap fraction has initially a much lower activity than either the whole liver or microsome protein, but the activity gradually increases with time,

Table 1. *Radioactivities of proteins isolated from the livers of guinea pigs killed at various time intervals after the intravenous injection of a mixture of [¹⁴C]amino acids*

L represents the protein of the complete liver; *S*, that of the cell-sap fraction; *M*, that of the washed microsome fraction and *A*, *B*, *C*, *D* and *E* the proteins of the various microsome subfractions obtained by salt extraction. Under the conditions used, 10^{-3} μc/mg. of carbon gives 1000 counts/min.

Time after injection (min.)	Radioactivity of protein (counts/min./cm.²)							
	L	*S*	*M*	*A*	*B*	*C*	*D*	*E*
2	15	6	56	15	44	48	58	63
5	45	23	128	67	60	182	133	124
15	85	46	220	177	108	445	183	185
30*	136	76	323	348	162	762	285	227
60	167	113	337	309	197	673	349	302
195	184	164	397	248	222	392	408	368

* Each value given, except that for *S*, is the mean of two values obtained in two separate experiments. The results obtained were in good agreement.

continuing to an appreciable extent after incorporation into the microsome protein has essentially ended. These results are in agreement with the results of Keller, Zamecnik & Loftfield (1954).

Incorporation of [^{14}C]amino acids into the proteins of microsome subfractions and characterization of subfractions by chemical analysis

Table 1 records also our results on incorporation of [^{14}C]amino acids into the proteins of the various microsome subfractions obtained by the application of the extraction procedure outlined in Fig. 1 and in the Experimental section. In Fig. 2, the values have been plotted so as to show the specific radioactivity of the protein of the various fractions relative to the activity of the whole microsome protein at corresponding time intervals. It has been found that, whereas changes in the specific activity of the amino acid mixture administered result in different absolute values for the radioactivities of different cell proteins, the ratios of the activities of the various proteins are essentially constant. The method of expression adopted in Fig. 2 will, therefore, largely eliminate variations in activity due to differences in dose. The values given in Table 2 for chemical analyses of the microsome subfractions are those obtained for a single liver preparation; other analyses have shown that the values given may, in general, be taken as typical.

Fraction A. The protein extracted by 0·14 M sodium chloride had, shortly after injection of amino acids, a radioactivity considerably lower than the whole microsome protein, but activity increased with time and at 30 min. this protein fraction was slightly more active than the whole microsome protein. Later, activity fell and at 195 min. was 60 % of the activity of the whole microsome protein.

This fraction contained about 10 % of the total nitrogen and 12 % of the protein of the washed-microsome fraction. No RNA or lipid-soluble phosphorus could be detected. Most of the material extracted by 0·14 M sodium chloride was present in the first of the two extracts (Fig. 1). The second extract contained only about one-third of the material present in the first.

Fraction B. The protein of the fraction extracted by M sodium chloride had (except at 2 min.) an activity approximately one-half of that of the whole microsome protein.

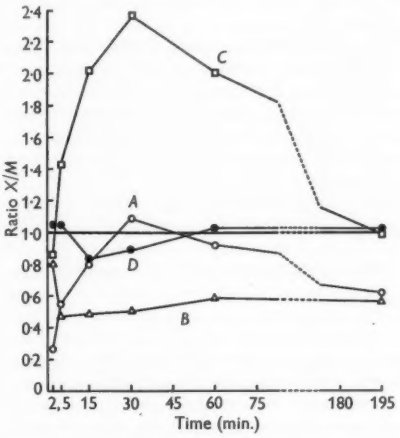

Fig. 2. Change of radioactivity of proteins of microsome subfractions with time after injection of radioactive amino acids. The radioactivity is expressed as the ratio of the specific activity of the subfraction protein (*X*) to that of the whole microsome protein (*M*). The curve for the protein of fraction *E* has not been given, as it was similar to that found for fraction *D*.

Table 2. *Analysis of fractions obtained from guinea-pig liver microsome material*

The fractions were prepared from a washed microsome fraction as described under Methods, except that a smaller quantity of NaOH was used to effect the separation of fractions *D* and *E* (see text).

	Percentage of complete microsome fraction				
Fraction	Total N	Protein	RNA*	Lipid-soluble P†	RNA‡ / Protein
A_1	7·6	9·9	—	—	—
A_2	2·4	2·4	—	—	—
B_1	9·9	10·1	18·2	Trace	0·21
B_2	3·4	3·2	6·7	Trace	0·24
C	11·8	12·3	43·2	Trace	0·41
D	11·4	14·1	21·4	8	0·17
E	53·6	48·0	10·4	89	0·024

* RNA values have been corrected for degradation occurring in cold perchloric acid (see text).

† Traces of lipid-soluble P appeared to be present in fractions B_1, B_2 and *C*, but the amounts were too small to be determined with precision.

‡ The RNA/protein ratio of the complete microsome material was 0·114.

This fraction contained about 13 % of the total nitrogen, 13 % of the protein, and in the experiment detailed in Table 2, 25 % of the RNA of the whole microsome fraction. The RNA/protein ratios were 0·21 for the first extract and 0·24 for the second. In other experiments, values of 0·60 and 0·45 have been obtained for the RNA/protein ratio of fraction *B* and the proportion of the total RNA in *B* was rather higher, the RNA value for fraction *C* then being correspondingly lower. The second M sodium chloride extract contained about one-third of the material found in the first. No more than trace quantities of lipid-soluble phosphorus were detected.

Fraction C. The protein of this fraction, extracted by buffer, pH 9, increased rapidly in radioactivity to a maximum value, more than twice that of the whole microsome protein, at about 30 min. The activity then fell rapidly and at 195 min. this fraction was no more active than the whole microsome protein.

Fraction *C* contained about 12 % of the total nitrogen, 12 % of the protein and 43 % of the RNA of the complete microsome fraction. The RNA/protein ratio was 0·41. In other experiments, ratios of 0·27 and 0·32 have been recorded. Lipid-soluble phosphorus was present only in trace quantities.

Fractions D *and* E. The proteins of these fractions (separated by solubility in 0·01 M sodium hydroxide) showed a similar pattern of labelling to one another. The initial rate of increase in activity was less than that for whole microsome protein, but after 30 min., as the activity of fraction *C* declined, these two fractions continued to increase in activity, and eventually they reached an activity approximately equal to the whole microsome protein.

Fractions *D* and *E* represent together about 65 % of the total nitrogen, 62 % of the protein, 32 % of the RNA and essentially all of the lipid-soluble phosphorus of the microsome fraction. The division between fractions *D* and *E* is somewhat arbitrary, depending upon the quantity of alkali added per unit weight of material. In the experiment recorded in Table 2, 0·6 ml. of 0·01 N sodium hydroxide/mg. of nitrogen was used; in other experiments 1·0 ml. was used, and a considerably greater proportion of lipid-soluble phosphorus and protein was brought into solution, approximately equal quantities of protein and lipid-soluble phosphorus being found in fractions *D* and *E*. With 0·6 ml. of 0·01 N sodium hydroxide/mg. of nitrogen, the RNA/protein ratio of fraction *D* was 0·17; with larger quantities of sodium hydroxide, the ratio was lowered. With progressive rise in the pH of the extractant, the remaining RNA is readily dissolved, together with increasing amounts of protein and lipid-soluble phosphorus, until only a small insoluble residue of protein remains (cf. Dallam, 1955).

Deoxyribonucleic acid

Neither the whole microsome fraction nor any of the fractions *B*, *C*, *D* or *E* when tested separately contained a quantity of DNA detectable by the method of analysis employed. Fraction *A* was not tested.

Characterization of microsome subfractions by physical methods

Ultraviolet-absorption curves. The ultraviolet-absorption spectra of the fractions (Fig. 3) confirmed the results of chemical analyses. The curve obtained for fraction *A* resembled that given by a protein solution, whereas curves found for fractions *B* and *C* indicated the presence of relatively large quantities of nucleic acid. Because of the characteristic differences found, the absorption spectra of the fractions were always determined, providing a useful means of following the progress of the fractionation procedure and checking on the nature of the extracts obtained.

Electrophoresis and sedimentation. Fractions *A* and *C* when analysed by electrophoresis at pH 8·4 as described in the Experimental section showed complex, though dissimilar, patterns, indicating the presence of numerous components. In the analytical ultracentrifuge, at pH 8·4, both *A* and *C* showed a rapidly moving peak (S_{20} = 7–8) and also a slower-moving more diffuse peak (S_{20} = 3–4). When fraction *B* was dialysed and freeze-dried, a substantial part of the material failed to redissolve in buffer (with fractions *A* and *C* almost all the freeze-dried material was soluble). As a consequence, it was difficult to obtain a solution of sufficiently

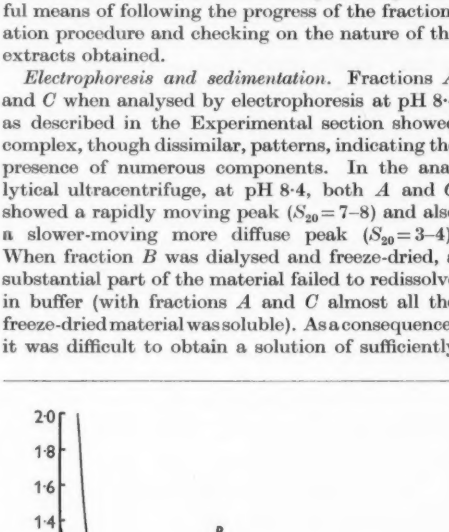

Fig. 3. Ultraviolet-absorption curves for microsome sub-fractions in aqueous solution.

high concentration for electrophoresis or ultracentrifuging. The pattern obtained in the ultracentrifuge at pH 8·4 suggested that the soluble material contained a large number of components of comparatively low molecular weight. No peak was found corresponding to the more rapidly moving component of the other two fractions.

DISCUSSION

Analytical methods. Standard methods of chemical analysis were quite satisfactory when applied to the whole microsome fraction, but difficulty was experienced when these methods were applied to the isolated microsome subfractions. Determination of RNA, by either ultraviolet absorption at 260 mμ. or estimation of phosphorus or pentose, showed that the RNA of the microsome subfractions was particularly labile and that upon contact with perchloric acid at 0° for 60 min. or less, some of the RNA was degraded into soluble fragments. In the experiment described in Table 2, this amounted to 26 % of the RNA. A small quantity of nucleotide material was extracted from the whole microsome fraction by cold perchloric acid (cf. Siekevitz, 1955), but this was much less than was obtained by similar extraction of a corresponding quantity of the microsome subfractions. In consequence, RNA values for fractions *B*, *C*, *D* and *E* have been calculated as the sum of the acid-soluble nucleotide plus the usual perchloric acid digest (RNA digest). This procedure appears to be justified, for in the values given in Table 2 the sum of the acid-soluble nucleotide plus the RNA digest nucleotide for fractions *B*, *C*, *D* and *E* was 97 % of the nucleotide content of the RNA digest of the whole microsome fraction.

Difficulty was also encountered in the isolation of protein from fraction *A*. Thus it was found that the protein of fraction *A* was largely soluble in 80 % (v/v) ethanol (method of Schneider, 1945) and was not reprecipitated completely on acidification after digestion with sodium hydroxide (method of Schmidt & Thannhauser, 1945).

Fractionation procedure. At first sight, the fractionation scheme (Fig. 1) used in this investigation may appear to be somewhat arbitrary, but preliminary experiments showed that, in fact, the conditions were rather critical and that the fractionation could be reproduced readily from one experiment to another. The success of the method depends upon the solubility characteristics of the various subfractions, and these differ rather sharply; thus a single extraction with 0·14 M sodium chloride extracted most of fraction *A* and a second extraction with the same solvent dissolved relatively little more protein. The distinction between fractions *B* and *C* depended almost entirely on control of pH, *B* being soluble in M sodium chloride at pH 7 (0·01 M phosphate buffer being used to control the pH). Further extraction at this pH or even at pH 8·3 (0·05 M bicarbonate buffer) with M sodium chloride dissolved little further material, but at pH 9 it was possible to extract fraction *C* with 0·1 M bicarbonate–carbonate buffer. As already indicated in the Results section, the separation between fractions *D* and *E* is much less well defined and they can hardly be regarded as distinct entities.

The present procedure is a development of a method first suggested by Dallam (1955), but the results obtained in the present investigation differ quantitatively from those which he reported. This difference is probably due to the different nature of our own and Dallam's subcellular fractions. Our guinea-pig liver microsome fraction was isolated by ultracentrifuging using 0·25 M sucrose, whereas the 'small-granule' fraction of Dallam was isolated with aqueous glycerol. The 'small-granule' fraction was isolated from 38 % (v/v) glycerol by centrifuging at 12000 ***g*** for 15 min. We have found that centrifuging for at least 40 min. at 30000 ***g*** was required for the complete sedimentation from 38 % (v/v) glycerol of the microsome fraction previously isolated from liver at 103000 ***g*** in 0·25 M sucrose. It is thus likely that a large part of the microsome fraction would not sediment from 38 % (v/v) glycerol at the centrifugal force employed by Dallam. The 'large-granule' fraction of Dallam was sedimented from 63 % (v/v) glycerol by centrifuging at 10000 ***g*** for 10 min. and it is likely that complete sedimentation of the mitochondrial fraction was not achieved; thus Dallam's 'small-granule' fraction would probably contain a considerable quantity of the mitochondrial fraction, and it is not surprising that in further fractionation of this material his results should differ substantially from those reported here.

Distribution of radioactivity between microsome proteins

The results of the present investigation confirm that in the normal animal the liver microsome protein has a much higher turnover rate than the protein of other liver fractions. Perhaps the most striking feature of the present data is the different behaviour of the nucleoprotein fractions *B* and *C*. Fraction *B* protein has a relatively low turnover rate, whereas the protein of fraction *C* reaches maximum activity 30 min. after injection of isotope and is at this time nearly five times as active as the protein of fraction *B*.

While this work was in progress, several quite different methods for the fractionation of microsome material were reported, and it is particularly instructive to compare the results of experiments on amino acid uptake made with these different

methods. Mirsky and his colleagues (Allfrey, Daly & Mirsky, 1953, 1955; Daly, Allfrey & Mirsky, 1955) have found that the protein released together with nucleotides when the microsome fraction of mouse pancreas is treated with ribonuclease has a higher turnover rate than the whole microsome protein; the time of maximum labelling of their fraction is about 1·5 hr. after administration of labelled amino acid. Hultin (1955) used prolonged extraction with 0·1 M bicarbonate at pH 8·4 to obtain a liver-microsome subfraction containing nearly all the RNA of the microsomes. The protein of this fraction was more rapidly labelled than that of any other fraction examined. Littlefield *et al.* (1955) treated rat-liver microsomes with deoxycholate, which dissolved much of the lipoprotein present, and they obtained a suspension of ribonucleoprotein particles with a rather similar RNA/protein ratio to that of our fraction *C*. The protein of the ribonucleoprotein granules (which accounted for most of the RNA of the microsomes) had a much higher turnover rate than the protein of the whole microsome fraction and reached its maximum activity only 3 min. after injection of radioactive amino acid.

It is thus clear that protein which is closely associated with RNA can be shown by four different methods to have a higher turnover rate than the protein of the whole microsome fraction. Each method provided a protein fraction with a different turnover rate, but each group of workers has used a different animal species and some of the differences recorded might be due to this. The time of maximum labelling varies over such a large range (3–90 min.), however, that another explanation seems more likely. In the present work, the protein associated with RNA was found to be heterogeneous; the apparent turnover rate of a mixture of different proteins will obviously vary widely according to the proportions in which those proteins are present and would no doubt change greatly with the use of different methods for extraction of ribonucleoprotein. This may well explain some of the divergent results.

Since the proteins of fractions *A* and *C* seem to have a higher turnover rate than the other liver proteins, it is tempting to suggest that they are precursors of other proteins; however, the demonstration that fractions *A* and *C* contain numerous components makes it impossible at present to provide much support for such a postulate, since the turnover rates of the individual components are unknown. It would obviously be desirable in work of this nature to study individual proteins, and some progress along these lines has been made by Daly *et al.* (1955). They found that trypsinogen and chymotrypsinogen of mouse pancreas showed a higher rate of incorporation of amino acid than did a protein fraction released from microsomes by ribonuclease, but in view of the present demonstration of the metabolic heterogeneity of microsome ribonucleoprotein their suggestion that microsome proteins are not precursors of trypsinogen or chymotrypsinogen is not necessarily valid.

Function of RNA in protein synthesis. The present work, together with other similar work already mentioned, suggests that in the normal liver cell the initial fixation of amino acids in peptide linkage takes place in a nucleoprotein fraction. Although a protein fraction with a very high rate of turnover and isolated by a variety of techniques was always associated with RNA, this does not necessarily imply that the RNA is required for protein synthesis. However, evidence has accumulated over the past 10 years that tissues with a high capacity for protein synthesis are rich in RNA (cf. Brachet, 1955), and various workers have reported that removal of RNA from whole cells or from subcellular particles by ribonuclease inhibits protein synthesis (for references see Askonas, Simkin & Work, 1956); moreover, Gale & Folkes (1955) have obtained a restoration of synthetic capacity in bacterial subcellular particles by addition of RNA or an RNA digest.

Bernhard, Gautier & Rouiller (1954) and Palade & Siekevitz (1956) have provided evidence that the liver-cell cytoplasm contains a structure, referred to as the ergastoplasm or endoplasmic reticulum, which consists of a series of convoluted membranes along which are disposed numerous small ribonucleoprotein particles. They suggested that most of the microsome fraction is derived from this structure. The present investigation demonstrates that the microsome fraction is metabolically heterogeneous. This may be accounted for, at least in part, by the heterogeneous nature of the microsome fraction itself. Kuff, Hogeboom & Dalton (1956) have emphasized that the microsome fraction may contain a variety of structures.

SUMMARY

1. Guinea pigs were killed at various time intervals after the intravenous injection of a mixture of ^{14}C-labelled amino acids (chlorella-protein hydrolysate). The liver-microsome fraction was separated into a number of subfractions of distinct composition by a procedure involving successive extraction with solutions of varying ionic strength and pH.

2. The proteins of the microsome subfractions were labelled at widely differing rates. The protein fraction with the greatest turnover rate was associated with ribonucleic acid. This protein showed a maximum activity about 30 min. after administration of labelled amino acids. Another microsome subfraction also contained ribonucleoprotein, but the protein of this fraction showed a much lower turnover rate.

3. The microsome subfractions were analysed for protein, ribonucleic acid, deoxyribonucleic acid and lipid-soluble phosphorus. Applying usual procedures for the separation of protein and nucleic acid, the ribonucleic acid of several subfractions was found to be abnormally unstable in perchloric acid. Several of the subfractions were examined in the ultracentrifuge and by electrophoresis.

4. The results have been compared with those obtained by other methods of fractionation of the microsomes, and the possible role that ribonucleic acid may play in protein synthesis is discussed.

We wish to thank Mrs F. Higginson and Miss M. T. Smith for skilled assistance; also Mr J. Orr for operating the ultracentrifuge, and Mr M. R. Young for phase-contrast microscopy. The Eli Lilly and Co. Research Fund supplied the Perkin–Elmer apparatus used in this investigation.

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The Breakdown of Adenosine Triphosphate Accompanying Cholic Acid Activation by Guinea-Pig Liver Microsomes

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It has previously been shown that microsomal preparations from guinea-pig liver form cholylhydroxamic acid from cholic acid in the presence of adenosine triphosphate (ATP), coenzyme A (CoA) and high concentrations of hydroxylamine (Elliott, 1955, 1956*a*). When taurine at substrate levels is added to the same system in place of hydroxylamine, taurocholic acid is formed and this reaction is also dependent on the presence of ATP and CoA (Elliott, 1956*b*). Bremer (1956) and Siperstein & Murray (1956) have independently reported the synthesis of taurocholic acid by microsome preparations. These observations indicate that cholyl-CoA formation is an intermediate step in taurocholic acid synthesis. The enzyme system bringing about the synthesis of cholyl-CoA will subsequently be referred to as the cholic acid-activating system.

The present paper is concerned with the breakdown of ATP which accompanies the activation of cholic acid by the cholic acid-activating system of guinea-pig liver microsomes. It is shown that adenosine 5′-phosphate (AMP) and inorganic pyrophosphate are formed. Some properties of the microsomal pyrophosphatase which complicated the investigation are also described.

EXPERIMENTAL

Hydroxylamine. Solutions of low KCl content were prepared by the method of Beinert *et al.* (1953). Norit A was kindly supplied by Dr D. E. Hughes. The inorganic phosphate present in the charcoal was removed by boiling in N-HCl for 15 min. and filtering with suction. The material was washed with water until the washings were neutral and dried at 105°.

ATP. This was obtained as the crystalline disodium salt (Schwarz Laboratories Inc.); solutions were adjusted to pH 7·4 with KOH.

Glyoxaline buffer. This was prepared by adjusting the pH of solutions of the free base with HCl. Measurements of pH were made at 37°.

Other preparations were as described previously (Elliott, 1956*a*, *b*).

Estimation of inorganic pyrophosphate. Two methods were used for this: first, a modification of the direct colorimetric method of Flynn, Jones & Lipmann (1954), and secondly, the determination of 10 min. acid-hydrolysable phosphate after removing nucleotides with charcoal (non-nucleotide P_{10}).

Colorimetric method

Samples (2·0 ml.) of incubation mixtures were pipetted into 2·0 ml. of 10% trichloroacetic acid and the precipitates removed by centrifuging. The supernatants were poured into centrifuge tubes and 200 mg. of washed Norit A was stirred into each tube. After 15 min. with continuous agitation, the charcoal was centrifuged off and the supernatants were poured into clean tubes and again centrifuged. Three successive centrifugings were needed to remove all the charcoal. Samples (0·5 ml.) of the final supernatants were used for the estimation of inorganic phosphate (P_i) and similar samples for the separate determination of P_i + pyrophosphate. For the latter a modification of the method of Flynn *et al.* (1954) was employed. These authors showed that Versene (ethylenediaminetetraacetic acid), present initially, caused a reduction in colour development. Use was made of this to stop the colour development at the appropriate time and so avoid the necessity of attempting to reach extinctions in solutions whose colour was rapidly increasing. After standing for 90 min., 1·0 ml. of 0·1 M ethylenediaminetetraacetic acid (EDTA) (as the sodium salt, pH 7·2) was added to each tube. Fig. 1 shows that this at once stops further development of colour and a very slow decrease then takes place. The extinctions were measured within 10 min. of adding EDTA. Internal standards of pyrophosphate were added to the unknowns.

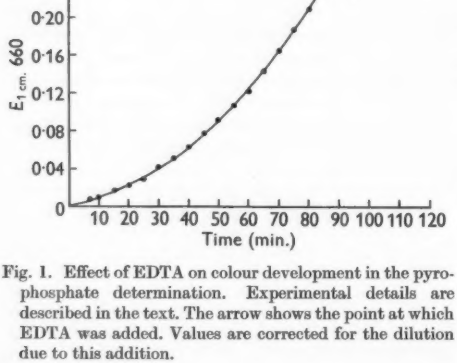

Fig. 1. Effect of EDTA on colour development in the pyrophosphate determination. Experimental details are described in the text. The arrow shows the point at which EDTA was added. Values are corrected for the dilution due to this addition.

Estimation of non-nucleotide P_{10}

Samples (0·5 ml.) of the supernatants from the charcoal treatment were mixed with 0·5 ml. of 2 N-HCl and heated at 100° for 10 min. and the total inorganic orthophosphate was estimated. After charcoal treatment it was found that there were no ultraviolet-light-absorbing spots on chromatograms of the supernatant fractions, showing that the phosphate formed on hydrolysis (P_{10}) was non-nucleotide in origin.

Inorganic orthophosphate. Estimations were by the method of Fiske & Subbarow (1925).

AMP. This was estimated chromatographically by the method of Krebs & Hems (1953). Samples (0·05 ml.) of the supernatants before charcoal treatment were applied to the paper.

Estimation of cholylhydroxamic acid. This was carried out as described previously (Elliott, 1956*a*), except that the standard curve from which values were taken was made by adding the standard synthetic cholylhydroxamic acid solution to 'control' incubation mixtures which did not contain cholic acid. The solutions were then treated in the same way as the experimental incubation systems. The curve obtained is thus corrected for the loss of hydroxamic acid in the protein precipitate; this amounts to 8·6% of the total.

Chromatography of inorganic pyrophosphate. Inorganic pyrophosphate was identified chromatographically in the charcoal-treated solutions. Samples (0·05 ml.) were spotted onto Whatman no. 1 papers washed with EDTA (Eggleston & Hems, 1952) and ascending chromatograms run for 18 hr. in a solvent consisting of di*iso*propyl ether (90 parts) and 90% formic acid (80 parts). Inorganic orthophosphate and inorganic pyrophosphate had R_F values of approximately 0·7 and 0·4 respectively when applied to paper on the same spot as a sample of the incubation mixtures. Pyrophosphate ran considerably slower under these conditions than when it was chromatographed by itself in the absence of incubation mixture.

RESULTS

Appearance of inorganic orthophosphate at low Mg^{2+} *ion concentration.* When the conditions of incubation previously found to be optimum for cholylhydroxamic acid synthesis (Elliott, 1956*a*) were used, inorganic orthophosphate was liberated from ATP. In the absence of cholic acid the amount formed was greatly reduced (Table 1). It can be seen that the phosphate release, above that due to ATPase activity, which was caused by the addition of cholic acid was equivalent to the amount of cholylhydroxamic acid formed. The apparent stoicheiometry between these two might be an artifact resulting from the partial hydrolysis of pyrophosphate, as indeed proved to be the case. The action of the microsome suspension on inorganic pyrophosphate was therefore investigated. In addition, Table 1 also shows that the omission of hydroxylamine, surprisingly, did not result in a diminished phosphate release, but rather that a slight stimulation occurred. This is discussed later.

Some properties of microsomal pyrophosphatase

Preliminary experiments showed that the microsome suspension hydrolysed added pyrophosphate and that this activity was reduced by the addition of KF. However, when tests were carried out for pyrophosphatase activity under the same conditions (except for the omission of ATP) as are used for the cholic acid-activating system, it was found that the rate of hydrolysis of pyrophosphate was not sufficient to account for the orthophosphate released during the synthesis of cholyl-CoA. The tests were carried out with a concentration of pyrophosphate of 1 μmole/ml., which is the greatest amount that could be expected to accumulate if pyrophosphate was liberated during cholyl-CoA synthesis. Moreover, additional experiments showed that the pyrophosphatase activity was not inhibited by this concentration of substrate, and in fact increased hydrolysis was observed up to the highest concentration of pyrophosphate tested (5 μmoles/ml.). Experiments were therefore carried out to see whether ATP stimulated microsomal pyrophosphatase, since this was the only component present in the cholic acid-activating assay system which was not present in the pyrophosphatase tests. Fig. 2 shows that a marked stimulation occurred, the activity in the presence of 0·005 M ATP being double that found with pyrophosphate alone. This is the smallest effect which has been found. With some preparations of microsomes, a fourfold increase in activity resulted from the addition of ATP. Adenosine diphosphate (ADP) and EDTA also stimulated the pyrophosphatase activity but were less effective than was ATP. AMP, orthophosphate and sulphate had no effect.

Effect of Mg^{2+} ion on microsomal pyrophosphatase in the presence and absence of fluoride. The effect of Mg^{2+} ion on the pyrophosphatase activity of microsomes is somewhat complicated. In the absence of fluoride, Mg^{2+} ion slightly increases the activity (Fig. 3), but it inhibits very strongly in the presence of fluoride (Fig. 4), complete inhibition being reached at a concentration of about 0·015 M-$MgCl_2$. It is possible that the stimulatory action of ATP and ADP [which have been shown by Smith & Alberty (1956) to form complexes with Mg^{2+} ion] and of EDTA, on pyrophosphatase activity in the presence of fluoride, is due to the formation of chelates with Mg^{2+} ion which lowers its effective concentration and consequently increases pyrophosphatase activity. These findings made it possible to suppress the inorganic pyrophosphatase activity of microsomes by increasing the concentration of Mg Cl_2 to 0·015 M. This had no effect on the cholic acid-activating system. Under these conditions ATP no longer stimulated the pyrophosphatase as it did when the concentration of Mg^{2+} ion was 0·005 M.

Table 1. *Liberation of inorganic orthophosphate at low Mg^{2+} ion concentration*

Incubations were carried out at 37° for 90 min. The complete system contained 0·1 ml. of 0·5 M glyoxaline buffer, pH 7·4, at 37°; 0·25 ml. of 0·1 M ATP; 1·25 ml. of 2 M hydroxylamine; 0·05 ml. of 0·5 M cysteine; 0·25 ml. of 0·5 M KF; 0·5 ml. of CoA (500 Lipmann units/ml.); 0·25 ml. of 0·1 M-$MgCl_2$; 0·25 ml. of 0·04 M potassium cholate; 1·0 ml. of washed microsome suspension. The volume was adjusted to 5·0 ml. with water. After incubation 2·0 ml. samples were pipetted into tubes containing 1·0 ml. of 7% (w/w) $HClO_4$ for hydroxamic acid determinations. Separate 2·0 ml. samples were measured into tubes containing 1·0 ml. of 10% (w/v) trichloroacetic acid, the protein was centrifuged off and inorganic orthophosphate determined in the supernatants. All values are expressed in terms of 2·0 ml. of incubation mixture.

Expt.	P_i produced (μmoles)	P_i release due to addition of cholate (1–2)	Cholyl-hydroxamic acid formed (μmoles)
1. Complete system	4·55	2·05	2·1
2. Cholate omitted	2·50	—	—
3. Hydroxylamine omitted	5·33	—	—

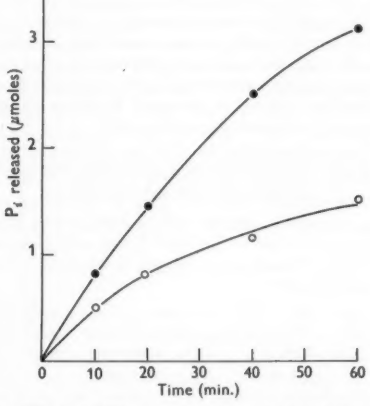

Fig. 2. Effect of ATP on microsomal pyrophosphatase. The complete system contained 0·1 ml. of 0·5 M glyoxaline buffer, pH 7·4, at 37°; 0·1 ml. of 0·1 M-$MgCl_2$; 0·1 ml. of 0·5 M-KF; 0·1 ml. of 0·1 M cysteine; 0·2 ml. of 0·01 M sodium pyrophosphate; 0·1 ml. of 0·1 M ATP; 0·3 ml. of washed microsome suspension; the volume was adjusted to 2·0 ml. with water. Incubations were carried out at 37° for 60 min. and stopped by the addition of 1·0 ml. of 10% (w/v) trichloroacetic acid; inorganic orthophosphate was estimated in the supernatants after centrifuging. All values are corrected for phosphate release which occurred in the absence of pyrophosphate. ●, With ATP; ○, no ATP.

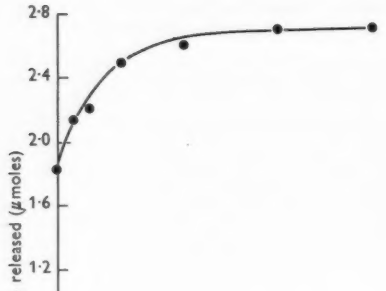

Fig. 3. Effect of Mg^{2+} ion on microsomal pyrophosphatase in the absence of fluoride. Incubations were carried out at 37° for 30 min. The complete system contained 0·1 ml. of 0·5M glyoxaline buffer, pH 7·4, at 37°; 0·1 ml. of 0·1M cysteine; 0·2 ml. of 0·01M sodium pyrophosphate; 0·3 ml. of washed microsome suspension. The volumes were adjusted to 2·0 ml. with water. After incubation 1·0 ml. of 10% (w/v) trichloroacetic acid was added to each tube, the protein centrifuged down and the inorganic orthophosphate determined in the supernatants. All values are corrected for the small P_i release which took place in the absence of pyrophosphate.

Table 2. *Measurement of orthophosphate release at high* Mg^{2+} *ion concentration*

Experimental conditions were as described in Table 1, except that 0·1 ml. of 0·3M-$MgCl_2$ was added instead of 0·1M-$MgCl_2$. Values are expressed in terms of 2·0 ml. of incubation mixture.

Expt.	P_i formed (μmoles)	Cholyl-hydroxamic acid formed (μmoles)
1. Complete	1·35	1·8
2. Cholate omitted	1·5	0·1

Formation of pyrophosphate at high Mg^{2+} *ion concentration*

Table 2 shows that when the level of Mg^{2+} ion is increased, the amount of orthophosphate which is released from ATP is no longer markedly stimulated by the addition of cholic acid. This phosphate release is presumably due simply to ATPase activity in the microsomes.

Table 3 shows, however, that a breakdown of ATP into AMP and inorganic pyrophosphate occurs under these conditions. The presence of pyrophosphate in Expt. 2 (Table 2) was also demonstrated by paper chromatography of the trichloroacetic acid supernatant after removal of nucleotides on charcoal. In the *iso*propyl ether–formic acid solvent there was a phosphate spot in the same position as that of authentic pyrophosphate added to the sample.

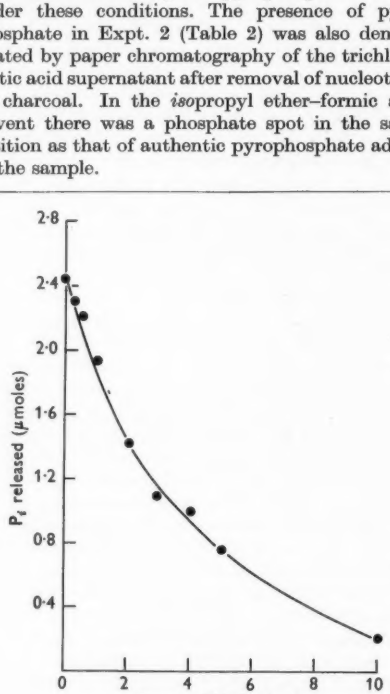

Fig. 4. Effect of Mg^{2+} ion on microsomal pyrophosphatase in the presence of fluoride. Experimental conditions were as described in Fig. 3, except that 0·1 ml. of 0·5M-KF was added and incubations were continued for 60 min.

Table 3. *Formation of inorganic pyrophosphate at high* Mg^{2+} *ion concentration*

Experimental conditions were as described in Table 2. The values are expressed in terms of 2·0 ml. of incubation mixture.

Expt.	Pyrophosphate (μmoles) determined by: Colorimetric method	Pyrophosphate (μmoles) determined by: P_{10} method	Cholyl-hydroxamic acid (μmoles)	AMP (μmoles)
1. Complete, stopped at zero time	0·25	0·43	0·25	3·1
2. Complete	4·0	4·25	2·1	6·1
3. Cholate omitted	1·72	1·98	0·35	4·07
Differences due to addition of cholate (2–3)	2·28	2·07	1·75	1·93

The results show an approximate stoicheiometry between the cholate-dependent formation of pyrophosphate, of AMP and of hydroxamic acid. An exact stoicheiometry between cholylhydroxamic acid and pyrophosphate production has not been obtained. In several experiments the amount of pyrophosphate liberated always exceeded that of cholylhydroxamic acid formed. Values differing as widely as 2·95 μmoles of pyrophosphate compared with 2·2 μmoles of hydroxamic acid have been obtained. This discrepancy has not been fully investigated but it may be related to the observation, described below, that a release of pyrophosphate, independent of hydroxylamine, occurs, which nevertheless requires the addition of cholic acid.

Formation of inorganic pyrophosphate in the absence of hydroxylamine. As already shown in Table 1, a release of orthophosphate occurred at low Mg^{2+} ion concentrations even in the absence of hydroxylamine. This release was found in subsequent experiments to be partly dependent on the addition of both CoA and cholic acid. High Mg^{2+} ion concentrations abolished the phosphate production but under these conditions a liberation of pyrophosphate occurs instead (Table 4). Little inorganic pyrophosphate is produced in the absence of CoA whether cholic acid is present or not. In the presence of CoA alone the production of pyrophosphate is about one-half that in the presence of both CoA and cholic acid.

Effect of taurine and glycine on pyrophosphate formation. Although pyrophosphate release occurs in the absence of hydroxylamine, it was possible that taurine, which is a 'natural acceptor' for cholyl-CoA (Elliott, 1956*b*), and possibly also glycine, would stimulate this release. Table 5 shows that this does not in fact occur, the production of pyrophosphate (measured as non-nucleotide P_{10}) being unchanged by addition of these amino acids.

Table 4. *Formation of inorganic pyrophosphate in the absence of hydroxylamine*

Experimental conditions were as described in Table 2, except that hydroxylamine was omitted. All values are expressed in terms of 2·0 ml. of incubation mixture.

Expt.	Pyrophosphate present* (μmoles)	P_i released
1. Complete	3·44	1·71
2. Complete, stopped at zero time	0·20	—
3. Cholate omitted	1·58	1·35
4. CoA omitted	0·48	1·5
5. Cholate and CoA omitted	0·22	2·0

* Measured as P_{10}, which was non-adsorbable on Norit A.

Table 5. *Effect of taurine and glycine on inorganic pyrophosphate formation*

Experimental conditions were as described in Table 2, except that hydroxylamine was omitted and 0·2 ml. of a 0·1 M solution of either taurine or glycine was added where appropriate.

Expt.	Pyrophosphate released* (μmoles)
1. No additions	2·0
2. With cholate	4·16
3. With cholate + taurine	4·20
4. With cholate + glycine	4·25

* Measured as P_{10}, which was non-adsorbable on Norit A.

Attempt to fractionate the cholic acid-activating system

A much clearer understanding of the various results would be obtained if the enzymes involved could be separated from one another to some extent. Attempts were therefore made to solubilize and fractionate the microsomal proteins. The addition of cholic acid or treatment of suspensions of the particles with ultrasonic vibrations yielded clear-yellow solutions whose cholic acid-activating system was not sedimented by centrifuging at 100000 *g* for 90 min. Attempts to fractionate these solutions with ammonium sulphate, heat treatment, etc., were not successful however. Very poor resolution into fractions was obtained and the precipitates so formed proved to be largely insoluble. In particular, no separation of inorganic pyrophosphatase activity from the cholic acid-activating system was achieved by any of the attempts. It seems probable that the 'solutions' obtained from the microsomes consisted of dispersions of very high molecular weight material rather than of true solutions of proteins.

DISCUSSION

The results show that the cholic acid-activating system of washed microsomes forms AMP and inorganic pyrophosphate from ATP in the course of converting cholic acid into cholyl-CoA. Although an exact stoicheiometry between pyrophosphate liberation and cholylhydroxamic acid formed has not been obtained it seems clear that the synthesis of cholyl-CoA proceeds by the following reaction:

$$\text{Cholic acid} + \text{ATP} + \text{CoA} \rightarrow \text{cholyl-CoA} + \text{AMP} + \text{inorganic pyrophosphate.}$$

It should be noted that pyrophosphate release in the presence of cholic acid and CoA does not depend upon the presence of hydroxylamine. In the absence of hydroxylamine the formation of inorganic pyrophosphate is dependent on the presence of CoA and partially dependent on the presence of

cholic acid. It seems reasonable to assume that that part of the pyrophosphate release which is dependent on both cholic acid and CoA is coupled with cholyl-CoA formation. The cause of that part of the pyrophosphate formation which is dependent on the addition of CoA only has not been investigated.

Since CoA is present in catalytic amounts only, it must be possible for cholyl-CoA to be converted back into CoA by a reaction other than that with hydroxylamine. This could conceivably occur by a reaction with glycine if this were present in washed microsomes, together with an enzyme system to condense the cholyl-CoA and glycine to form glycocholic acid. Previous work (Elliott, 1956*b*) has, however, shown that no taurocholic acid synthesis occurs in the absence of added taurine, and it seems unlikely that there could be sufficient glycine present to saturate an acceptor system using this amino acid. This point has not been investigated however. More probably there is a cholyl-CoA deacylase which catalyses the hydrolysis of cholyl-CoA as follows:

$$\text{Cholyl-CoA} + H_2O \rightarrow \text{cholic acid} + \text{CoA}.$$

Enzymes of this type have been found by Schachter & Taggart (1953), hydrolysing benzoyl-CoA, and by Gergely, Hele & Ramakrishnan (1952), hydrolysing acetyl-CoA and succinyl-CoA.

Such a system might well account for the observation that, in the presence of hydroxylamine, the pyrophosphate release is somewhat in excess of cholylhydroxamic acid formation. If the reaction of the cholyl-CoA with hydroxylamine is insufficiently rapid then some of the cholyl-CoA would be broken down by the deacylase and inorganic pyrophosphate would be produced without any corresponding production of hydroxamic acid.

The inhibition of microsomal pyrophosphatase by Mg^{2+} ion in the presence of fluoride, but not in its absence, has not been reported previously so far as the author is aware. Whether the reason for the effect is the existence of two pyrophosphatases, or the formation of an inhibitory complex involving Mg^{2+} ion and fluoride, is not known. The results suggest that, at any rate in the presence of fluoride, effects of compounds on pyrophosphate levels in experiments with microsomes should be interpreted with caution, in case the effect is an indirect one due to chelation of a metal ion and consequent change in the rate of pyrophosphate breakdown.

The activation of cholic acid occurs by a reaction analogous to those for acetic acid (Lipmann, Jones, Black & Flynn, 1952), short-chain fatty acids (Mahler, Wakil & Bock, 1953) and long-chain fatty acids (Kornberg & Pricer, 1953), involving the breakdown of ATP to AMP and inorganic pyrophosphate. The enzyme system activating long-chain-fatty acids occurs in microsomes as well as in the soluble fraction (Kornberg & Pricer, 1953). It cannot be decided from the present work whether the cholic acid-activating system is the same as or different from the system activating long-chain fatty acids.

The reaction by which taurocholic acid is formed from cholyl-CoA is similar to that for hippuric acid formation (Schachter & Taggart, 1954), though the latter system is localized in the mitochondria and not in the microsomes. The synthesis of the peptidic bonds of glutamine (Speck, 1949; Elliott, 1951), and of the two peptide bonds of glutathione (Mandeles & Bloch, 1955; Snoke, Yanari & Bloch, 1953), proceed by quite different mechanisms which do not involve CoA and result in the breakdown of ATP to ADP and inorganic orthophosphate. Among the small peptides which accumulate in reasonably large amounts in animal tissues the only ones which have not been synthesized in an *in vitro* system would now appear to be carnosine and its methylated derivative anserine.

SUMMARY

1. At low concentrations (0·005 M) of Mg^{2+} ion the synthesis of cholyl-coenzyme A by guinea-pig liver microsomes is accompanied by a release of inorganic orthophosphate from adenosine triphosphate (ATP). This release is abolished by Mg^{2+} ion at a concentration of 0·015 M.

2. The inorganic pyrophosphatase of microsomes is strongly inhibited by Mg^{2+} ion in the presence, but not in the absence, of fluoride. ATP, adenosine diphosphate and ethylenediaminetetraacetic acid can activate the enzyme, probably by chelating Mg^{2+} ion, provided that fluoride is present.

3. At a Mg^{2+} ion concentration of 0·015 M, cholylhydroxamic acid synthesis is accompanied by a release of inorganic pyrophosphate, though it has not been possible to demonstrate an exact stoicheiometry between them. The release of pyrophosphate is not dependent on the presence of hydroxylamine. Possible explanations of these results are discussed.

4. The synthesis of oligopeptides in animal tissues is briefly discussed.

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The Nature of the Fluctuating Ribonucleic Acid in *Escherichia coli*

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In several species of bacteria growing cells have been shown to have much higher concentrations of ribonucleic acid (RNA) than resting cells. Apart from the work of Belosersky and his collaborators (Belosersky, 1947) there has been little investigation into the nature of the 'additional RNA' in growing cells; more attention has been paid to its possible function in the cell. The most widely accepted view, that it plays a part in the synthesis of protein, is based mainly upon the direct relationship observed between the RNA concentration and the growth rate of micro-organisms growing exponentially (Caldwell, Mackor & Hinshelwood, 1950; Price, 1952; Wade, 1952; Jeener, 1953), upon the presence of RNA in granules having the ability to synthesize proteins (Gale & Folkes, 1955*a*) and upon the concomitant syntheses of protein and RNA which occurs during enzyme induction (Pardee, 1954). Some experimental results, however, do not lend themselves to this theory, and there is increasing evidence that the level of RNA concentration does not reflect the rate at which a cell is synthesizing protein.

The contrasting RNA concentrations in resting cells and growing cells is more pronounced in some species than in others. In *Escherichia coli* the concentration of RNA in rapidly growing cells is approximately twice the concentration in resting cells, and in certain other species more than fivefold differences in concentration have been observed. The abundance of the 'additional RNA' in fast-growing bacteria and the conflicting evidence presented for its connexion with protein synthesis prompted the closer examination of its nature and function in *Esch. coli*.

It was important in the first instance to establish whether or not the increased RNA concentration which develops in resting cells during the early stages (division lag) of their growth in fresh medium is due to a general increase in the concentration of all RNA constituents or to only a fraction of these. To this end the chemical compositions, the solubilities, the responses to various methods of extraction and the sedimentation behaviours of ribonucleic acids in resting and in dividing cells of *Esch. coli* were compared. Concurrently with these experiments the effects of various extractions and fractionations upon the basophilia of the cells were observed in order to examine the relationship between the 'additional RNA' and the high basophilia of dividing cells. This report describes these experiments and suggests an alternative function for 'additional RNA' based upon our results and upon other published experimental data.

A preliminary note on part of this report has already been published (Wade, 1955).

MATERIALS AND METHODS

Organisms and cultural conditions. Comparisons between the RNA concentrations in resting and in dividing cells were made on *Aerobacter aerogenes* (NCTC 418), *Bacillus anthracis* (Weybridge), *Bacillus cereus* (NCTC 8035), *Chromobacterium prodigiosum* (laboratory strain), *Chromatobacterium violaceum* (NCTC 7917), *Clostridium welchii* (SR 12), *Corynebacterium hoffmannii* (NCTC 8338), *Eberthella typhosa* (Ty 479, a purine-requiring avirulent mutant kindly supplied by Dr T. W. Burrows), *Escherichia coli* (NCTC 1100), *Pasteurella pestis* (Tjiwidej), *Proteus vulgaris* (laboratory strain) and *Staphylococcus aureus* (Oxford 'H') grown on casein–casein–yeast medium (CCY) (Gladstone & Fildes, 1940) or in tryptic-meat broth (Table 1) at 37°.

The cultures were grown aerobically with the exception of *Cl. welchii* and *P. vulgaris* which were grown anaerobically in screw-capped bottles on tryptic-meat broth containing 0·5% of glucose and 0·08% of thioglycollic acid. Resting cells were taken from fully grown cultures and dividing cells from cultures during the phase of maximum growth and division rate. The growth rate of dividing cells was assessed from the increase in optical density, which was measured in 1 cm. cells of the Hilger Spekker absorptiometer with a neutral filter (Hilger, 508) and, within the limits of the range *E* 0·05–0·5, to which measurements were confined, was found to be proportional to the dry-weight concentration of the cells. The average cell size was assumed to be constant during this phase of growth. The cultures were treated with 4% (w/v) formaldehyde soln., the cells were centrifuged, washed once with 20 vol. (wet cell vol.) of 0·145 M-NaCl and once with 20 vol. of water.

An investigation into the nature of *Esch. coli* RNA was carried out on resting cells and dividing cells from aerated CCY cultures grown at 37°. Each culture was cooled rapidly to 0–5°, centrifuged in a DeLaval continuous centrifuge at 2–5°, and the cells were washed twice with 20 vol. of 0·145 M-NaCl at 2–5°.

Cell suspensions. Suspensions of viable cells were prepared by suspending 1 vol. of washed cells in 2 vol. of 0·145 M-NaCl. Acid-extracted cells were prepared by suspending the cells in 10 vol. of 5% (w/v) trichloroacetic acid (TCA) at 0–5°. After standing overnight the cells were centrifuged, washed once with 10 vol. of 5% TCA and finally with 10 vol. of water. The cells were taken up into 1 vol. of water, and IRA-400 (OH) ion-exchange resin (British Drug Houses Ltd.) was added to remove remaining traces of TCA. Ethanol-extracted cells were prepared by delivering the saline suspension of viable cells into 10 vol. of ethanol at 0–5°. The cells were allowed to sediment from the resulting suspension and resuspended in water or 0·145 M-NaCl.

Extraction of cells. The intact cells, prepared by one of the methods described above, were extracted with at least 10 vol. (wet cell vol.) of extracting agent for 30 min. at room temperature (Table 3). Determinations of RNA carried out upon the suspension and upon the supernatant after centrifuging at 1500 *g* for 15 min. gave a measure of the RNA extracted. Changes in the physical state of the cells and basophilia were also noted.

The solubility of RNA between pH 0·3 and 8 in alkali-treated suspensions of *Esch. coli* was determined in the following way. Approximately 2 g. wet wt. of washed, viable cells was mixed with 50 ml. of water and the suspension treated with 50 ml. of 0·1 N-NaOH. After 5 min. at 20°, 10 N-HCl was added, with vigorous stirring, to pH 8. A sample of 5 ml. was withdrawn and stored at 0°. The pH was brought progressively lower by further additions of 10 N-HCl and, at suitable intervals, further samples were taken. RNA-pentose determinations (described below) were carried out on the sample taken at pH 8 and on the supernatants of all samples obtained by centrifuging at 1000 *g* for 15 min. at 20°.

Ultrasonic disintegrations. These were carried out in flat-bottomed flasks of 150–200 ml. capacity which were prepared with bases of 0·25–0·5 mm. thickness and approximately 6 cm. diam. Water suspensions, prepared by the addition of 9 vol. of water to washed cells, were used in preference to saline suspensions since disintegration was more rapid in this medium. The flask containing the suspension was supported by a retort stand 1–2 cm. above the transducer of a Mullard ultrasonic generator producing vibrations of 2 Mcyc./sec. with an energy output of 500 w. Both the transducer and the bulb of the flask were immersed in an ethanol bath refrigerated to −10°. The position of the flask was frequently adjusted to obtain the maximum turbulence. The temperature of the suspension did not rise above 7° during disintegration. Similar water suspensions were disintegrated by shaking with glass beads (Mickle, 1948) after the addition of octanol to reduce foaming. Periods of continuous disintegration at 2–5° were limited to 10 min., after which the vessels were removed and cooled in iced water. The temperature did not rise above 10° during this disintegration. Disintegrations were continued until less than 0·1% of whole cells remained; this was normally accomplished in 1–2 hr.

Fractionation of disintegrates by centrifuging. This is summarized in Fig. 1. A volume (30 ml.) of each disintegrate was centrifuged at an average centrifugal force of 1000 *g* for 30 min. at 2–5°. Approximately 25 ml. of the supernatant was centrifuged at an average centrifugal force of 25 000 *g* for 1 hr. at 0° in a Spinco model L centrifuge (no. 40 rotor) and approx. 12·5 ml. of this supernatant was centrifuged at an average centrifugal force of 100 000 *g* for 4 hr. at 0°. Chemical determinations and the assessments of basophilia were carried out on the suspensions of the deposits in 0·145 M-NaCl and on the final supernatant (*S* III). Sedimentation analyses of the disintegrates were carried out in the Spinco model E centrifuge. The sedimentation coefficients were corrected to water at 20° and a partial specific volume of 0·75 ml./g. was assumed for all the components except deoxyribonucleic acid (DNA), for which a value of 0·55 ml./g. was used (Peacocke & Schachman, 1954).

Analysis of phosphorus, nitrogen and magnesium. The methods described by King (1951) were used for the colorimetric determinations of phosphorus and nitrogen. These determinations were carried out in triplicate and the colour intensities were measured on the Hilger Spekker absorptiometer.

Magnesium was determined spectrographically. In order to obtain samples and standards in similar solutions, the protein present in each sample was removed by precipitation with an aqueous solution of TCA and propan-2-ol. Samples (0·1 ml.) containing magnesium (20–400 p.p.m.) and 0·1 ml. of chromium soln. (250 p.p.m.) were delivered into 4·8 ml. of aqueous solution of 5% (w/v) TCA and 10% (v/v) propan-2-ol. The mixture was allowed to stand for 5 min. and the precipitated protein removed by centrifuging. A carbon electrode impregnated with the protein-free sample or with a standard solution containing magnesium (1–20 p.p.m.)

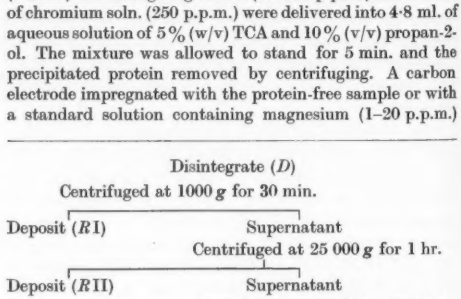

Fig. 1. Fractionation of disintegrates.

and a counter electrode were sparked for 30 sec. with a Hilger condensed-spark set. The spectrum produced by a Hilger large quartz spectrograph was recorded on an Ilford orthochromatic plate and the intensities of the 2803 Å magnesium and 2836 Å chromium lines were measured with a non-recording microphotometer. The determinations were carried out in triplicate; three pairs of electrodes were prepared for each sample and standard. Carefully standardized conditions were used throughout and an accuracy of ±5% was obtained.

Estimation of RNA. The concentration of RNA in bacteria was determined by the reaction of orcinol and $FeCl_3$ with acid hydrolysates of acid-extracted cells (Morse & Carter, 1949), yeast RNA (Light Co. Ltd.) containing 8·7% of phosphorus being used as standard. Samples of the hydrolysate were dried down before their reaction with orcinol in the manner described below.

RNA pentose (acid-insoluble purine-bound pentose) was determined in the following manner. Each sample, containing 5–40 μg. of RNA pentose, was delivered into 3 ml. of 5% (w/v) TCA at room temperature and, after 30 min., was centrifuged and drained free from supernatant. The residue was dried at 60° under negative pressure from a water pump. Samples taken from the TCA extract for the determination of acid-soluble pentose were similarly treated. Chromatographically pure ribose (Light Co. Ltd.) standards were prepared with each set of determinations. A volume (0·5 ml.) of orcinol reagent (Militzer, 1946) was delivered into each tube and the tubes were immersed in a boiling-water bath for 10 min. During this period each tube was shaken briefly to mix the pad of dried material which was hydrolysing. The tubes were then cooled rapidly in cold water and 4·5 ml. of *n*-butanol was delivered into each of them. The determinations were carried out in triplicate, and the colour intensities read on an Hilger Spekker absorptiometer with a red filter (Hilger 608). A standard curve was drawn with each set of determinations and the RNA pentose or acid-soluble pentose determined from it. RNA nitrogen was calculated from the RNA-pentose concentration and the ribonucleotide ratio of *Esch. coli* RNA (RNA nitrogen × 1·52 = RNA pentose).

Nucleotide ratios. The ribonucleotide ratios of RNA in resting cells and dividing cells of *Esch. coli* were determined by the ionophoresis of alkaline hydrolysates. Cells extracted with TCA and ethanol–ether-extracted cells (Schmidt & Thannhauser, 1945) were hydrolysed in 0·3 N-NaOH for 18 hr. at 37° (Crosbie, Smellie & Davidson, 1953) and the ribonucleotides formed were precipitated as their barium salts and prepared for ionophoresis (Wade & Morgan, 1955) by the method described previously for the analysis of adenosine triphosphate preparations (Wade & Morgan, 1954*b*). The separations were conducted for 4 hr., after which period the mixed nucleoside 2′- and 3′-phosphates of cytosine, adenine, guanine and uracil had moved approximately 2·0, 3·3, 5·2 and 7·3 cm. respectively. The nucleotides were detected and determined by a method described previously (Wade & Morgan, 1954*b*).

Dry weight determinations. These were made directly on washed cells dried at 105° for 18 hr. (Wade, 1952).

Basophilia estimates. The basophilia of sedimentation fractions was assessed with the aid of Oxoid membrane filters (Oxo Ltd., London). An area 6 cm. × 3 cm. was supported on six 9 cm. diam. Whatman no. 50 filter papers supported on a Büchner funnel. Suction from a water pump was applied and 2 ml. of 0·33 N-HCl delivered on to the surface of the membrane followed by 2 ml. of water. Volumes of the fractions were then applied to different positions on the membrane; acid-insoluble basophilic constituents of the fractions were precipitated by the residual acidity and retained on its surface. The deposits were washed successively with 2 ml. of 0·33 N-HCl and 2 ml. of water by passing them through the membrane. The latter was removed, soaked in toluidine blue soln. at pH 3·5 (Wade & Morgan, 1954*a*) for 20 min. at 20°, returned to the pile of filter papers and washed thoroughly with water to remove unbound dye.

The development of 'additional RNA' was followed in resting cells of *Esch. coli* inoculated into tryptic-meat broth at 37°. Samples taken at intervals were treated with formaldehyde to a concentration of 1% formaldehyde. The suspensions were centrifuged and the cells washed once with 1% formaldehyde soln. The cells were taken up into a little residual supernatant and applied to a slide. The smears were air-dried, extracted with 0·33 N-HCl for 10 min., washed with water, stained for 15 min. at 45° with toluidine blue soln. (Wade & Morgan, 1954*a*), rinsed with water, air-dried, stained with eosin soln. (Wade & Morgan, 1954*a*) for 50 sec., then rinsed with water and air-dried.

RESULTS

Concentrations of ribonucleic acid in the cells of a number of bacterial species. The RNA concentrations in the resting cells and dividing cells examined are set out in Table 1. Since the method adopted for this determination depends upon the colour formation of only purine-bound pentose, differences between the ribonucleotide compositions of RNA from different species influenced the values obtained and did not therefore permit interspecific comparisons of these values to be made. Evidence presented below, however, suggested that the ribonucleotide compositions of RNA from resting cells and dividing cells are similar, and justified a comparison between the ratios of RNA concentration in the resting and dividing cells of different species. These ratios (Table 1) varied markedly from species to species and did not reflect similar variations in the growth rates of the dividing cells.

Ribonucleotide ratios of RNA in resting cells and dividing cells of Esch. coli. The results of four determinations are set out in Table 2. They revealed no significant differences between the RNA in the two types of cell. This result enabled a rough determination of the ribonucleic acid nitrogen to be calculated from the RNA pentose (RNA nitrogen × 1·52 = RNA pentose) and applied to extracts and fractions of the cell.

Extraction of RNA from intact cells. The concentration of RNA in dividing cells of *Esch. coli* is approximately twice that in resting cells. If the 'additional RNA' in dividing cells is less strongly bound (Belozersky, 1947), and can be extracted from intact cells, this would result in the removal of

Table 1. *RNA concentration in the cells of a number of bacterial species*

Organism (1)	Medium* (2)	Doubling time† (min.) (3)	RNA/dry wt. (%) Resting cells (4)	RNA/dry wt. (%) Dividing cells (5)	RNA ratio (5)/(4) (6)
Aerobacter aerogenes	CCY	30·9	4·4	26·6	6·0
Bacillus anthracis	TMB	48	1·45	24	16·5
Bacillus cereus	TMB	23	3·5	31·5	9·0
Chromobacterium prodigiosum	CCY	28·5	7·8	32·1	4·1
Chromobacterium violaceum	TMB	60	7·2	30·3	4·2
Clostridium welchii	TMB	42·8	32·2	42·2	1·3
Corynebacterium hoffmannii	CCY	128	25·4	51	2·0
Eberthella typhosa	CCY	38·7	11·3	32·4	2·9
Escherichia coli	CCY	27·3	15·6	34·5	2·2
Pasteurella pestis	TMB	116	5·9	14·9	2·5
Proteus vulgaris	TMB	27·3	12·6	35	2·8
Staphylococcus aureus	CCY	30	5·2	10	1·9

* CCY, casein–casein–yeast medium; TMB, tryptic-meat broth.
† Period of twofold increase in optical density during the logarithmic growth of dividing cells.

approximately 50 % of the total RNA from dividing cells without any removal from resting cells. The results of various extractions carried out on viable, acid-extracted and ethanol-extracted cells are summarized in Table 3.

In water, viable dividing cells lysed more rapidly than resting cells. Both types of cell lysed rapidly in mild alkali but, in the presence of NaCl, mild alkali extractions could be carried out without disrupting them. However, by the use of 2 M-NaCl and different degrees of alkalinity conditions could not be found by which RNA was extracted specifically from dividing cells in the quantity expected from Belosersky's theory. Furthermore, the ratio RNA nitrogen/total nitrogen of these extracts approximated to 0·2, which suggested the presence of a large proportion of other nitrogenous substances.

Acid-extracted cells retained their physical state for several months in water at 2–5°. Suspensions in water or saline gave a weak acid reaction with indicators. Upon neutralization with sodium bicarbonate, suspensions both of resting cells and of dividing cells lost RNA to approximately the same extent; small differences in pH about neutrality had a marked influence upon the quantity removed (Table 3).

Ethanol [5–50 % (v/v)] extracted RNA to approximately the same extent from both resting and dividing cells (Table 3). Ethanol-extracted dividing cells lost a greater proportion of RNA when subsequently extracted with water or 0·145 M-NaCl than resting cells. However, when the progress of this extraction was followed over a longer period this difference was found to be only transitory.

In all instances where substantial removal of RNA from dividing cells was effected, the loss of basophilia was also observed (Table 3). In some instances it was possible to demonstrate this on a

Table 2. *Ribonucleotide ratios of RNA relative to adenylic acid from resting cells and dividing cells*

Condition of cells	Molar ratio of ribonucleotides: Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
Resting	1·01	1·0	1·32	0·99
Resting	0·98	1·0	1·36	0·93
Dividing	0·95	1·0	1·31	0·94
Dividing	0·87	1·0	1·36	0·93

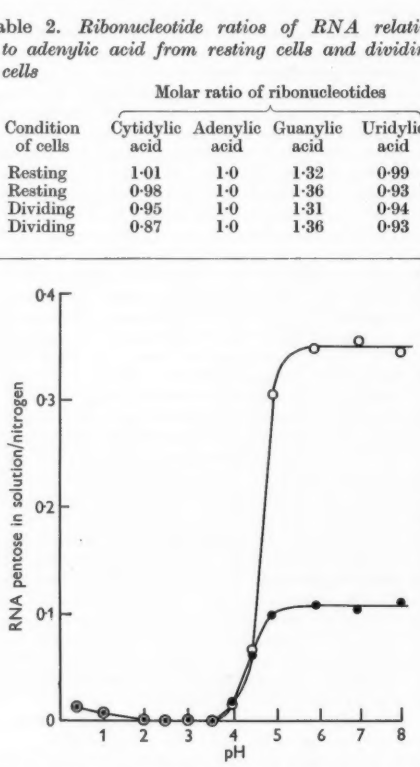

Fig. 2. Solubility of RNA of alkali-treated *Esch. coli*. For details of method used see text. ○, Resting cells (total RNA concn. = 0·165 RNA pentose/N); ●, dividing cells (total RNA concn. = 0·412 RNA pentose/N).

microscope slide. When acid-extracted cells were dried on a slide and treated with 0·145 M-NaCl containing sufficient sodium bicarbonate to neutralize the suspension the dividing cells lost their high basophilia and could then be distinguished from resting cells only by their larger size. A similar effect was obtained by treating acid-extracted cells with 1 % (v/v) Tergitol 7 (British Drug Houses Ltd.) at neutral pH.

Solubility of RNA in alkali-treated Esch. coli *cells.* The influence of pH upon the solubility of RNA in alkali-treated suspensions of resting and dividing cells is illustrated in Fig. 2. There was no indication of heterogeneity in the RNA from dividing cells and the RNA in both cells was completely precipitated at pH 2·5–3·5.

Acid-soluble nucleotide content of Esch. coli *cells.* Rough assessments of the ribonucleotides present in resting and dividing cells were obtained from the pentose contents and the optical densities at 260 mμ. of 5 % TCA extracts of these cells. The results, set out in Table 4, suggest that resting cells and dividing

Table 3. *Extraction of RNA from (A) viable, (B) acid-extracted and (C) ethanol-extracted* Esch. coli *cells*

Extracting agent	% RNA extracted		RNA nitrogen* / total N		Removal of basophilia from dividing cells
	Resting cells	Dividing cells	Resting cells	Dividing cells	
(A) Viable cells					
Water	0	Lysis	.	.	.
0·01 N-NaOH	Lysis	Lysis	.	.	.
0·01 N-NaOH + 0·14 M-NaCl	10·0	22·0	.	.	.
0·01 N-NaOH + M-NaCl	4·9	21·0	.	.	.
0·01 N-NaOH + 2 M-NaCl	2·1	20·0	.	.	.
0·1 M-$NaHCO_3$/Na_2CO_3 (pH 9·5) + 2 M-NaCl	0	7·5	.	0·05	−
0·01 N-NaOH + 2 M-NaCl	4·5	22·2	0·01	0·19	+
0·05 N-NaOH + 2 M-NaCl†	5·9	27·0	0·27	0·2	+
10 % Tergitol	0	0	.	.	−
1 % Sodium tauroglycocholate	0	0	.	.	−
5 % (v/v) ethanol	9·1	19·2	.	.	.
10 % (v/v) ethanol	7·0	16	.	.	.
25 % (v/v) ethanol	5·5	13·5	.	.	.
50 % (v/v) ethanol	1	2·9	.	.	.
(B) Acid-extracted cells					
Water	0	0	.	.	−
Water, adjusted to pH 6·5	54·2	72·0	0·6	0·83	+
0·14 M-NaCl	0	0	.	.	−
0·14 M-NaCl, adjusted to pH 5·5	5·1	18·3	.	.	−
0·14 M-NaCl, adjusted to pH 6·5	53·0	56·5	0·92	0·95	+
0·14 M-NaCl, adjusted to pH 7·5	87·0	100·0	.	.	+
0·1 M-$MgCl_2$, adjusted to pH 6·5	12·0	13·9	0·03	0·067	−
0·01 N-NaOH	Lysis	Lysis	.	.	.
0·05 N-NaOH + M-NaCl†	69	78	0·31	0·41	+
0·05 N-NaOH + 2 M-NaCl†	20	28	0·36	0·51	+
1 % CTAB, adjusted to pH 6·5	Lysis	Lysis	.	.	.
1 % Teepol, adjusted to pH 6·5	.	Lysis	.	.	.
1 % Tergitol 7, adjusted to pH 6·5	89	99	0·95	1·05	+
(C) Ethanol-extracted cells					
Water	7·8	47·5	0·05	0·57	+
0·14 M-NaCl	23·5	64·5	0·12	0·26	+

* $\frac{\text{Ribonucleic acid nitrogen}}{\text{Total acid-insoluble nitrogen}}$.

† Extracted for 30 sec. and then neutralized.

Table 4. *Concentration of pentose compounds soluble in 5 % trichloroacetic acid and substances absorbing at 260 mμ. in resting and dividing cells of* Esch. coli

Extracted material	Pentose extracted (mg.) / Cell N (mg.)		Optical density at 260 mμ. ($E^{\frac{1 \text{ mg. cell N/ml.}}{1 \text{ cm.}}}$)	
	Resting cells	Dividing cells	Resting cells	Dividing cells
Whole cells	0·029	0·022	2·35	2·06
Disintegrates	0·040	0·042	2·56	2·95

Table 5. *Distribution of nitrogen and RNA pentose in resting and dividing cells of* Esch. coli

M, cells disintegrated by the Mickle method; US, cells disintegrated by exposure to ultrasonic vibrations.

Fraction	Nitrogen distribution		RNA-pentose distribution		RNA nitrogen/total nitrogen (%)	
	M	US	M	US	M	US
Resting cells						
D	100	100	17·0	17·1	11·2	11·2
*R*I	4·5	1·3	1·0	0·2	14·7	10·1
*R*II	33·0	18·7	3·8	3·4	7·6	12·0
*R*III	25·4	28·1	8·3	7·7	21·7	18·7
*S*III	35·8	46·9	2·9	5·0	5·3	7·0
Dividing cells						
D	100	100	36·2	37·3	23·8	24·6
*R*I	7·3	1·9	2·0	0·2	18·1	6·9
*R*II	16·2	11·6	3·5	2·7	14·2	15·4
*R*III	49·0	42·7	26·6	26·5	35·7	41·2
*S*III	23·1	36·9	4·0	6·7	11·4	11·9

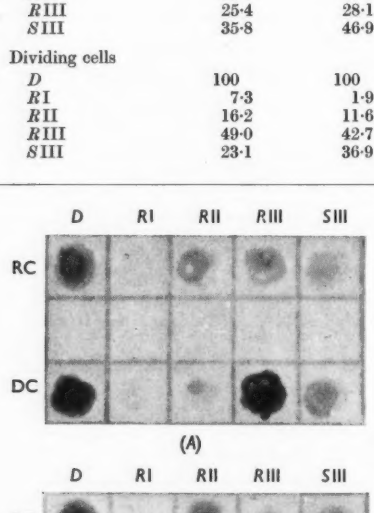

Fig. 3. Qualitative assessment of basophilia in sedimentation fractions of cell disintegrates obtained by (*A*) the Mickle method and (*B*) exposure to ultrasonic vibrations. RC, Resting cells; DC, dividing cells; *D*, unfractionated disintegrate; *R*I, deposit from 1000 *g*; *R*II, deposit from 1000–25 000 *g*; *R*III, deposit from 25 000–100 000 *g*; *S*III, supernatant from 100 000 *g*. The quantities applied were equivalent to approx. 25 μg. of N unfractionated disintegrate. Magnification ×2.

cells do not have widely different concentrations of these constituents.

Fractionation of Esch. coli *disintegrates by centrifuging.* The course of this fractionation is given in Fig. 1. The results of nitrogen and RNA-pentose determinations carried out on samples of *D* and *S*III and also on *R*I, *R*II and *R*III after resuspension in 0·145 M-NaCl are presented in Table 5.

The results showed that the increase in RNA concentration which occurs during the division lag of *Esch. coli* is due to an increase in RNA-containing constituents which sediment at 25 000–100 000 *g* and not to an increase in all RNA-containing constituents. In both disintegrates, one prepared by the Mickle method (M-disintegrate) and the other by exposure of the cells to ultrasonic vibrations (US-disintegrate), the difference between fractions *R*III of resting cells and dividing cells accounted for the difference in the total RNA of the two types of cell.

The analyses (Table 5) also showed that the distribution of cell constituents between sedimentation fractions is influenced by the method used to disrupt the cells. The significantly smaller values for nitrogen and RNA-pentose in fractions *R*I and *R*II and the greater values in fractions *S*III from disintegrates of either resting or dividing cells disrupted by ultrasonic vibrations suggests that a greater fragmentation of the larger particles is brought about by this method.

A qualitative assessment of basophilia showed that the contrasting basophilia of resting cells and dividing cells (Fig. 3*D*) was due to a highly basophilic constituent of *R*III in dividing cells (Fig. 3, *R*III). In the course of this assessment it was observed that fractions *R*I and *R*II of both resting and dividing cells were metachromatic in reaction, unlike other fractions. This metachromatism, first observed during the drying of the stained fractions on the membrane, was readily demonstrated by the addition of small quantities of the suspended fractions to a weak aqueous solution of toluidine blue.

The magnesium contents of sedimentation fractions from M-disintegrates of resting cells and

dividing cells are set out in Table 6. The total concentration of magnesium in dividing cells was approximately 50 % greater than in resting cells; fraction *R*III of dividing cells was mainly responsible for this difference.

Sedimentation characteristics of resting-cell and dividing-cell disintegrates. An examination of the sedimentation patterns of disintegrates and fractions *S*III revealed marked differences between resting and dividing cells and also between the two methods of disintegration. The sedimentation pattern obtained from resting cell/M-disintegrate [Fig. 4 (*A*) (*a*)] was composed of two main peaks, 3·8 *S* and 10·8 *S*; the sharp nature and the sedimentation rate of the latter suggested it to be deoxyribonucleic acid (DNA). The pattern obtained from dividing cell/M-disintegrate [Fig. 4 (*A*) (*b*)] had three additional peaks, 19·8 *S*, 27·1 *S* and 34·7 *S*, only traces of which could be detected in the disintegrate of resting cells. The pattern from resting cell/US-disintegrate was composed of one major peak, 3·8 *S* [Fig. 4 (*B*) (*a*)]; that from dividing cell/US-disintegrate had two additional peaks, 20·6 *S* and 29·4 *S* [Fig. 4 (*B*) (*b*)] the sedimentation constants of which approximated to two of the peaks of the dividing cell/M-disintegrate. The constituents responsible for the additional peaks in the patterns of dividing-cell disintegrates were not present in the final supernatants *S*III [Fig. 4 (*A*) (*c*); (*B*) (*c*)].

Location of 'additional RNA' in dividing Esch. coli *cells.* The strongly basophilic character of the 'additional RNA' permitted its development in freshly inoculated resting cells to be followed under the microscope. It was observed that this RNA developed evenly throughout the cell during the division lag phase, a period of approximately 40 min., and that subsequently, during the period of maximum growth and division rates when RNA concentration was maximum, there was no marked difference between the concentrations in fully grown and in newly divided cells.

Table 6. *Distribution of magnesium in Mickle disintegrates of* Esch. coli

Fraction	Magnesium (g.)/100 g. of cell N	Mg (μg.)/mg. of N	Mg (μg.)/mg. of RNA pentose
Resting cells			
*R*I	0·14	31·7	151
*R*II	0·29	9·2	80
*R*III	0·35	14·2	44
*S*III	1·1	30·7	380
Dividing cells			
*R*I	0·38	52	189
*R*II	0·29	18·8	87
*R*III	0·82	17·5	31·6
*S*III	1·34	58·0	335

DISCUSSION

The RNA concentration in micro-organisms is known to vary with the physiological state of the cell, and a number of independent observations on several species of bacteria have suggested a

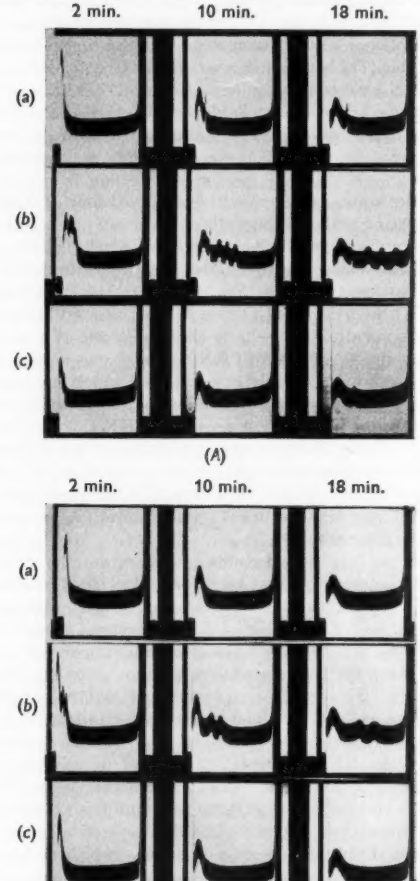

Fig. 4. Sedimentation patterns of disintegrates obtained by (*A*) the Mickle method and (*B*) exposure to ultrasonic vibrations. RC, Resting cells; DC, dividing cells. (*a*) RC disintegrate, (*b*) DC disintegrate, and (*c*) DC supernatant, after centrifuging at 100 000 *g* for 4 hr. (*A*) RC disintegrate, 1·5 mg. of N/ml.; DC disintegrate, 1·41 mg. of N/ml. (*B*) RC disintegrate, 1·31 mg. of N/ml.; DC disintegrate, 1·27 mg. of N/ml. Photographs were taken at 2, 10 and 18 min. after a maximum speed of 59 780 rev./min. had been reached.

correlation between RNA concentration and growth rate. The results set out in Table 1 provide qualitative evidence for this; the fast-growing cells have a higher concentration of RNA than the non-growing cells of the same species. For reasons already given an interspecific comparison of these values cannot be made; however, the evidence obtained from *Esch. coli* that the ribonucleotide compositions of ribonucleic acids from resting and dividing cells are similar (Table 2) justifies a comparison of the ratios of RNA concentration in resting and dividing cells. Such a comparison (Table 1) reveals the absence of a direct quantitative relationship between the changes in the RNA concentration and growth rate.

The similar ribonucleotide compositions of RNA in resting and dividing cells of *Esch. coli* provides no information on the heterogeneity of the RNA in dividing cells. However, the analyses of four sedimentation fractions (Fig. 1, *R*I, *R*II, *R*III and *S*III) have revealed (Table 5) that the 'additional RNA' in dividing cells is the result not of a proportional enrichment of RNA in all fractions but of an enrichment in only one of these, which suggests that the 'additional RNA' does not have a composition widely different from that of RNA present in resting cells. The similar compositions of RNA from sedimentation fractions of resting cells of *Esch. coli* observed by Elson & Chargaff (1955) appears, therefore, to extend to the 'additional RNA' present in dividing cells.

It has not been possible to obtain details of the experiments reported by Belosersky (1947) which are claimed to demonstrate that the 'additional RNA' in dividing cells of *Esch. coli* is not bound to protein, but the results of experiments carried out to examine this possibility do not support this theory. The acid-precipitation of 0·05 N-NaOH-treated cells of this bacterium (the conditions used for an initial 'extraction' by Belosersky) showed that the RNA of both resting and dividing cells behaved alike in this respect. Furthermore, not one of several different extraction techniques (Table 3) examined would remove free RNA specifically from intact dividing cells in a quantity expected from this theory. The possibility that preliminary treatment of the dividing cells analysed by Belosersky resulted in a partial degradation of the RNA is suggested by the analyses of TCA extracts (Table 4), the results of which do not confirm his observation that young cells (dividing cells) have a much higher proportion of mononucleotides than resting cells. However, the possibility was further entertained that free RNA, originally present, combined with protein upon extraction or disintegration of dividing cells. In one experiment the concentration of RNA in a disintegrate of resting cells was doubled by the addition of yeast sodium ribonucleate and the mixture centrifuged at 100 000 ***g***. Although 50 % of the added RNA was sedimented with the deposit, none of the additional peaks present in the sedimentation patterns of dividing-cell disintegrates (Fig. 4) was formed, suggesting that the RNA-rich macromolecular particles of dividing cells occurred naturally and were not artifacts of naturally free RNA.

Although it was not possible to demonstrate free RNA in cell disintegrates, it was possible to remove RNA from both acid-extracted resting cells and dividing cells practically free from other nitrogenous substances (Table 3). The ability to carry out these extractions on a microscope slide and observe the removal of basophilia under the microscope confirms the identity of the basophilic constituent of these cells (Belosersky, 1947; Tulsane & Vendrely, 1947). Unlike Tulsane & Vendrely (1947) and Onisi & Kato (1955), however, who revealed central basophilic bodies after they had removed the RNA by ribonuclease treatment or 0·145 M-NaCl extraction, no central bodies were revealed by this treatment.

There have been few reports describing the sedimentation patterns of bacterial disintegrates. Schachman, Pardee & Stanier (1952) and Billen & Volkin (1954) have described patterns which resemble those reported here. The dissimilar conditions employed prevent a direct comparison of sedimentation coefficients, but the identity of the particulate fraction (deposited at 160 000 ***g*** for 1 hr., after centrifuging at 7000 ***g*** for 10 min.) reported by Schachman *et al.* (1952), with fraction *R*III (Fig. 1) from dividing cells, is suggested by the similar RNA concentrations in these fractions (Table 5).

Considered together, the sedimentation patterns and chemical analyses of dividing cells disintegrated by the two methods described yielded some interesting information on the chemical nature of the fastest-moving component. Disintegration by exposure to ultrasonic vibrations resulted not only in the absence of the DNA peak from the sedimentation patterns, an effect expected from the work of Laland, Overend & Stacey (1952), but also in the gross reduction of the fastest-moving component of the Mickle-disintegrated dividing cells. [Compare Fig. 4 (*A*) (*b*) with Fig. 4 (*B*) (*b*).] The chemical analyses (Table 5), however, revealed no great difference between the RNA in fractions *R*III of the dividing-cell disintegrates obtained by the two methods, and suggested that the fastest-moving component of the Mickle-disintegrated dividing cells contained little if any RNA.

Although chemical analyses suggested a composition of approximately 41 % of RNA (Table 5) for the two constituents which sediment at about 20*S* and 28*S*, it is possible that the 'additional RNA' in dividing cells is composed of new constituents, which, in dividing-cell fraction *R*III (US) (Table 5),

are present in addition to those of resting-cell fraction *R*III (US) and that fractions 20*S* and 28*S* have, in fact, an average composition of approximately 85 % of RNA.

The biological significance of the 'additional RNA' in growing micro-organisms is not understood. The low rate of RNA-phosphorus (Cohen, 1948) and RNA-purine (Koch, Putnam & Evans, 1952; Manson, 1953) turnovers reported in phage-infected *Esch. coli* and in normal cultures (Hershey, 1954), has led to the belief that RNA is an inert by-product (Manson, 1953; Pardee, 1954), but such demonstrations of inactivity do not influence the general opinion that RNA is concerned in protein synthesis. The template theory of protein synthesis (Haurowitz, 1949; Dounce, 1952) does not depend upon a turnover of RNA and, as experiments by Hershey (1954) have shown, in growing cells of *Esch. coli* the phosphorus turnover of RNA, although small, is significantly greater than that of DNA.

The view that quantitative differences in RNA concentration are associated with differences in rate of protein synthesis stems chiefly from studies of metazoan cells which have shown that cells actively engaged in protein synthesis are rich in RNA (see reviews by Davidson, 1947; Caspersson, 1947; and Brachet, 1947). In micro-organisms the only positive evidence for this has been obtained from cells growing and dividing exponentially (Caldwell *et al.* 1950; Price, 1952; Wade, 1952; Jeener, 1953), a correlation which had been noted earlier between total nucleotide concentrations and rates of protein synthesis (Malmgren & Heden, 1947). This correlation has not been observed in other phases of growth. During the first generation of growth no correlation was observed between the growth rates and nucleotide concentrations (Malmgren & Heden, 1947) or RNA concentration (Wade, 1952), and the coincidence of the maximum concentration of RNA during this period with the middle of the first generation, has suggested that the varying concentration of RNA is more closely associated with the division process (Wade, 1952), an opinion also expressed by Davidson (1953) and Brachet (1955). The absence of a correlation between RNA concentration and growth rate was also observed during growth subsequent to exponential growth (Jeener, 1952*a*), during the growth stimulation of cells in nutritionally limiting medium (Abrams, Hammarsten, Reichard & Sperber, 1949; Jeener, 1952*b*), and during growth inhibition by cobalt ions (Levy, Skutch & Schade, 1949). Evidence from micro-organisms growing exponentially, however, is unsound, for under these conditions of growth the RNA concentration can be correlated with the rate of any process which, like the rate of protein synthesis, is proportional to the growth rate. Less equivocal evidence is needed before fluctuations in RNA concentration can be attributed to changes in protein synthesis.

It is possible to resolve some of the conflicting views on RNA function by assuming the presence of at least two RNA constituents in living cells, each with a distinct role. It is possible that whereas the concentration of one form ('constitutional RNA') remains constant and is directly concerned with protein synthesis, the concentration of another ('fluctuating RNA') varies directly with the rate of some other process which, during exponential growth, is directly related to the growth rate.

A number of demonstrations that the microsome fraction of the cell has protein-synthesizing ability (Hultin, 1950; Keller, 1951; Siekevitz, 1952; Allfrey, Daly & Mirsky, 1953; Keller, Zamecnik & Loftfield, 1954; Oota & Osawa, 1954; Gale & Folkes, 1955*a*, *b*) suggest that the RNA associated with this fraction is the 'constitutional RNA'. The distribution of RNA pentose in sedimentation fractions of *Esch. coli* (Table 5) is consistent with this theory; the aggregate fraction '*R*I plus *R*II', which contains particles similar in size to microsomes, is present in similar concentrations in both resting and dividing cells. The constancy of a particulate RNA fraction in yeast cells has been reported before by Brachet & Jeener (1943). The dependence of the maximum potential rate of protein synthesis upon this concentration is suggested by the results of Allfrey *et al.* (1953), who compared tissues of widely different metabolic activities and associated differences in microsome concentrations with differences in rates of protein synthesis.

Sedimentation data from *Esch. coli* suggest that, unlike the larger microsome particles of 'constitutional RNA' which are of the order 60–160 mμ. diameter (Davidson, 1953), the 'fluctuating RNA' exists in particles of the order 10 mμ. diameter. This RNA probably corresponds to the volutin-RNA of Belozersky (1947), the '20–40*S*' fraction of Schachman *et al.* (1952) and most of the 'soluble ribonucleoprotein' of Brachet & Jeener (Brachet & Jeener, 1943, 1944; Brachet, 1947). The function of such an abundant cell constituent merits a little speculation. RNA has been found in chromosomes (Frolova, 1944; Kaufmann, McDonald & Gay, 1948; Turchini, 1949), and its role in nuclear division (Brachet, 1947; Kaufmann & Das, 1954; Jacobson & Webb, 1952; Ledoux & Baltus, 1954) and cellular division (Thomas, Rostand & Gregoire, 1946; Wade, 1952) has been postulated. The association of RNA-rich particles with division in *Esch. coli* (Table 5) and in regenerating liver cells (Petermann, Mizen & Hamilton, 1953; Porter, 1954) has also suggested a function in the division process. It is tempting, therefore, to correlate the fact that a high concentration of magnesium is

associated with the 'fluctuating RNA' in fraction *R*III of dividing cells (Table 6) with the fact that a controlled deficiency of this element inhibits division in certain bacteria (Webb, 1949), and to postulate that this RNA is directly concerned with the division process, and that under conditions of magnesium deficiency its normal formation is prevented and the process it governs is inhibited. The dependence of the division process upon the strongly basophilic 'fluctuating RNA' would also account for the influence of several basic compounds which, under certain conditions, inhibit division but not growth, e.g. proflavine (Davies, Hinshelwood & Pryce, 1944), penicillin (Gardner, 1940), streptomycin (Stenderup, 1953), sulphonamide (Tunnicliff, 1939), methyl violet (Ainley-Walker & Murray, 1904), triethylenemelamine and ethyleneimine (Loveless, Speorl & Weissman, 1954).

The cytological observation that 'fluctuating RNA' is as abundant in fully grown cells as it is in newly divided cells suggests that it is not catabolized during the division process. The possibility of it being a source of energy, however, cannot be discredited by this observation since it may function by transferring energy in the manner postulated by Dounce (1952). Another possibility is that it plays a part in DNA synthesis. The failure of most tracer studies (Villee, Lowens, Gordon, Leonard & Rich, 1949; Abrams, 1951; Mitchell & Moyle, 1953; Hershey, 1954) to demonstrate a conversion from RNA into DNA (Brachet, 1947; Mitchell, 1942, 1943; Caldwell & Hinshelwood, 1950; Argell, 1952) could be attributed to the low concentrations of 'fluctuating RNA' present in the systems examined. The little information available at present leaves both possibilities equally acceptable and justifies their further entertainment.

SUMMARY

1. The analyses of resting and dividing bacterial cells have shown that in each of twelve species examined a higher concentration of ribonucleic acid (RNA) is present in dividing cells than in resting cells. A comparison of different species has further shown that the growth rate and the ratio of these concentrations are not related in a simple manner. The nature of the 'additional RNA' in dividing cells of *Escherichia coli* has been studied in greater detail.

2. The ribonucleotide compositions of RNA in resting cells and dividing cells are similar.

3. Attempts to extract the 'additional RNA' specifically from intact dividing cells were unsuccessful.

4. The analyses of sedimentation fractions from cell disintegrates have shown that the 'additional RNA' is present in a fraction (*R*III) which is sedimented at 25 000–100 000 *g*. The difference between the RNA content of this fraction from dividing cells and the same fraction from resting cells accounts for the difference between the total RNA concentration in these cells.

5. Depending upon the method used for disrupting the cells, fraction *R*III from dividing cells contains two or three prominent constituents with different sedimentation characteristics; only low concentrations of these constituents are present in resting cells.

6. Constituents of fraction *R*III are responsible for the difference in basophilia between resting cells and dividing cells and partly responsible for the higher concentration of magnesium in dividing cells.

7. The possibility of a connexion between the division process and the fluctuations in 'additional RNA' normally observed in batch cultures is discussed in the light of these results and other experimental data.

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The Interconversion of Serine and Glycine: Preparation and Properties of Catalytic Derivatives of Pteroylglutamic Acid

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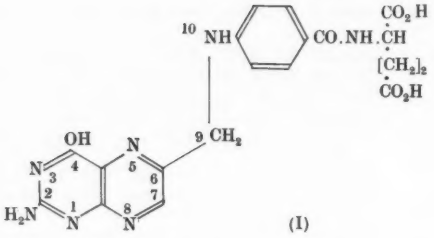
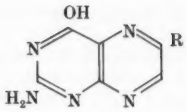
The catalytic activity of hydrogenated forms of pteroylglutamic acid (PGA; I) in the enzymic synthesis of serine is now well established (Blakley, 1954; Kisliuk & Sakami, 1955; Alexander & Greenberg, 1955). The mechanisms involved are obscure, but a possible mechanism is the enzyme-catalysed combination of hydrogenated PGA with formaldehyde to form a relatively stable intermediate. This intermediate may then react with glycine under the influence of a second enzyme to produce serine and regenerate hydrogenated PGA.

Many attempts have been made in this laboratory to isolate such a pteridine intermediate, since its structure would be the key to the mechanism of enzyme reactions involving PGA derivatives. Furthermore, if sufficiently stable, it might conceivably have pharmaceutical applications. Among difficulties encountered has been the heterogeneity and instability of the pteridines isolated, and this has led to a reinvestigation of the nature and stability of hydrogenated derivatives of PGA. Evidence is presented that most preparations of



(I)



(II)

(*a*) R = OH
(*b*) R = CH_3
(*c*) R = CO_2H

hydrogenated PGA contain, or readily form, several fluorescent components, and that most of these undergo profound oxidative degradation to products such as xanthopterin [II (*a*)] with little regeneration of PGA. In addition, experiments are reported which indicate the formation of freely dissociating compounds of formaldehyde and hydrogenated PGA, but not of stable condensation products.

EXPERIMENTAL

Materials

Paper chromatography revealed that all commercial samples of PGA were heavily contaminated with fluorescing material. All but a very small fraction of the fluorescing substances could be removed by repeated extraction of a solution of the PGA in phosphate buffer (approx. 0·5M, pH 5·5) with *n*-butanol. After five extractions with 3 vol. of butanol, the PGA was crystallized from the solution by acidification and recrystallized once in the same way, the crystals being washed twice with cold water in each case.

Chromatographically pure xanthopterin was generously provided by Professor A. Albert of this University. Gifts of 2-amino-4-hydroxy-6-methylpteridine and 2-amino-4-hydroxypteridine-6-carboxylic acid (chromatographically pure) were kindly provided by Dr H. P. Broquist of Lederle Laboratories.

The enzyme preparation was the same as that described previously (Blakley, 1955).

Methods

Determination of coenzyme activity. Estimation of the amount of material in pteridine preparations which was catalytic in the enzymic synthesis of serine was carried out under the standard conditions previously described (Blakley, 1955), with an excess of enzyme purified to the ammonium sulphate or to the ethanol fractionation stage. Since the enzyme deteriorated considerably on keeping, no direct comparison is possible between absolute activities in different experiments.

Hydrogenation of pteridines. This was carried out in Warburg flasks in most cases. Normally the platinum oxide catalyst (5 mg.) and 2·5 ml. of solvent were placed in the main compartment and the PGA (10 μmoles) dissolved in 0·5 ml. of solvent was placed in the side arm. The flask was placed on the manometer in the bath (37°), filled with H_2, and shaken till H_2 uptake ceased (about 1 hr.). The PGA solution was then tipped into the main compartment and shaking continued until the theoretical amount of H_2 had been consumed.

In larger-scale experiments in which the hydrogenated material was subsequently allowed to oxidize and the products were isolated, hydrogenation was carried out in a flask or tube connected by flexible tubing to a gas burette. The platinum oxide catalyst (equal in weight to the PGA to be hydrogenated) together with a small volume of solvent was placed in the vessel and the latter evacuated and filled with H_2. The vessel was evacuated and filled with H_2 twice more, and then shaken until H_2 uptake ceased. The PGA, dissolved in a further small volume of solvent, was then introduced under a stream of N_2 and the vessel again evacuated and filled with H_2 three times. Before shaking was recommenced and whenever subsequent readings of the gas burette were made the gas in the flask was first brought to a standard temperature and to atmospheric pressure. Shaking was continued until the theoretical amount of H_2 had been consumed.

Chromatography. Paper chromatography was carried out by the ascending technique on Whatman no. 4 sheets or strips and the pteridine spots were located over an ultraviolet lamp. Aromatic amines were detected by the modification of Ekman's reaction employed by Dalgliesh (1952). For a rapid indication of the nature of a pteridine mixture 0·1M-K_2HPO_4 was commonly employed as solvent, since it gave fair separation of the pteridines concerned in about 2 hr. Other solvent systems used for more exhaustive examinations were as follows: 5% (v/v) acetic acid; 0·1N-Na_2CO_3; 3% (w/v) NH_4Cl; 5% (w/v) boric acid; propan-1-ol–water–NH_3 soln. (sp.gr. 0·88) (200:99:1, by vol.); *sec.*-butanol–formic acid–water (8:2:5, by vol.); and the upper layer of a mixture *n*-butanol–acetic acid–water (4:1:3, by vol.).

Paper chromatography under anaerobic conditions was performed in a cylindrical glass vessel 53 cm. × 6 cm. fitted with a ground-glass socket at the lower end and drawn out to a thin tube at the upper. A 40 cm. × 5 cm. strip of Whatman no. 4 paper was placed in the vessel and suspended by a cotton thread. While a rapid stream of N_2 was passed through the vessel and over the chromatograph paper, solutions of the samples to be chromatographed were removed in capillaries from the Warburg vessels under a stream of N_2 and transferred to the chromatograph paper as it lay in the vessel with N_2 passing over it. When the paper had dried in the N_2 stream both ends of the vessel were closed. The pressure in the vessel was then reduced slightly so that O_2-free 0·1M-K_2HPO_4 could be sucked in through a stopcock in the lower end of the vessel until the solvent reached the paper. When the chromatogram had developed sufficiently the solvent was blown out with N_2 and the paper dried *in vacuo* and examined over the ultraviolet lamp. The partial pressure of O_2, although low, was not zero during the development of the chromatogram since leucomethylene

blue when subjected to chromatography in this way was largely oxidized to the blue dye by the time the chromatogram had developed.

For column chromatography Solka Flok (powdered cellulose, 200 mesh; Brown Co., 500 Fifth Avenue, New York, N.Y.) was employed as adsorbent in a glass column 110 cm. long × 5·5 cm. internal diam. The height of the column of Solka Flok was approx. 75 cm. Elution was performed with 9·6% (w/v) $(NH_4)_2CO_3$ or with 5% (v/v) acetic acid at a rate of about 200 ml./hr. and the eluate was collected in 20 ml. fractions. A column of this size satisfactorily handled oxidation products arising from 0·1 m-mole of hydrogenated PGA. Elution of these compounds was followed by measuring the optical density at 250 mμ. of fractions of eluate.

Determination of diazotizable amine. The method employed was a modification of that of Kaselis *et al.* (1951). The solution of the sample (volume up to 0·5 ml., containing 10–100 μg. of *p*-aminobenzoylglutamic acid) was placed in a test-tube to which was added 2·0 ml. of N-HCl and 2·0 ml. of 95% (v/v) ethanol. After mixing, 1·0 ml. of 0·1% (w/v) $NaNO_2$ was added and 2 min. later 1·0 ml. of 0·5% (w/v) ammonium sulphamate. After 3 min., 1·0 ml. of 0·1% (w/v) *N*-ethyl-1-naphthylamine in ethanol was added and the solution thoroughly mixed. The solution was made up to a volume of 10·0 ml. with 0·15N-HCl and the optical density at 520 mμ. determined 20 min. after adding the *N*-ethyl-1-naphthylamine.

A standard curve prepared from solutions of authentic *p*-aminobenzoylglutamic acid showed a linear relationship between optical density and amount of amine over the range 10–100 μg. of *p*-aminobenzoylglutamate.

Absorption spectra. Measurements were made with a Unicam spectrophotometer, silica cells being used.

Reaction of NN′-diphenylethylenediamine and formaldehyde. A portion (418 mg.; 1·97 m-mole) of recrystallized *NN′*-diphenylethylenediamine (m.p. 62°) was dissolved in 25 ml. of ethanol and 1·9 ml. of 1·25M formaldehyde (2·36 m-mole) added. The precipitate which formed was redissolved by adding a further 50 ml. of ethanol and warming. The solution was then allowed to stand overnight at room temperature, and the precipitate filtered off and recrystallized from aqueous ethanol. This first crop of material weighed 189 mg. and melted sharply at 123·6° (uncorr.). Further crops of crystals brought the total yield to 377 mg. (85% of the theoretical). Elementary analysis of a recrystallized sample gave the following results: C, 80·9; H, 7·23; N, 12·4. (Calc. for $C_{15}H_{16}N_2$: C, 80·4; H, 7·19; N, 12·5%.)

RESULTS

When identical amounts of PGA were simultaneously hydrogenated in Warburg flasks under the conditions described above but with various solvents, the reaction proceeded as shown in Fig. 1. As may be seen, hydrogenation proceeded most rapidly when PGA was hydrogenated in neutral aqueous solution (approx. pH 7), H_2 uptake quickly proceeding to a point where a little more than 2 moles had been consumed/mole of PGA. Hydrogenation ceased after a further small uptake.

Hydrogenation in 98% formic acid proceeded almost as rapidly as in neutral aqueous solution and likewise proceeded to an uptake of approx. 2 moles/mole of PGA. In 0·1N-NaOH hydrogenation was slower than in neutral aqueous solution, especially after an uptake of 1 mole of H_2/mole of PGA. Hydrogenation will continue beyond this point, however, and with much more vigorous shaking than can be obtained with the Warburg apparatus 2 moles of H_2 are absorbed/mole of PGA without difficulty. Even in Warburg flasks, however, the uptake eventually approaches close to 2 moles/mole of PGA (Fig. 1). Hydrogenation with acetic acid as solvent proceeded very slowly, doubtless owing to the fact that PGA is only slightly soluble in this solvent and must be added to the vessel as a suspension, whereas it dissolves readily in the other solvents investigated. With a weight of platinum oxide equal to that of PGA and in an apparatus permitting very vigorous shaking the PGA ultimately dissolved completely in acetic acid at the point when 2 moles of H_2 have been consumed/mole of PGA.

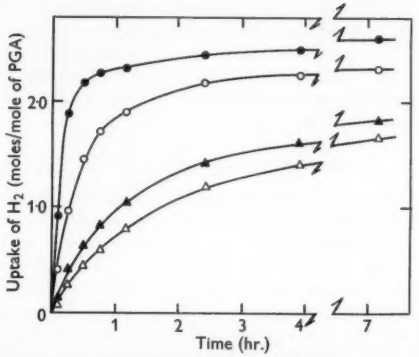


Fig. 1. Rate of hydrogenation of PGA in various solvents. ●, In 2 equiv. of aqueous $NaHCO_3$, final soln. pH 7; ○, in 98% (v/v) formic acid; ▲, in 0·1N-NaOH (10 mg. of catalyst); △, in 0·1N-NaOH (5 mg. of catalyst). For conditions see text.

Hydrogenation of analogues of PGA

Since the hydrogenation of PGA in 0·1N-NaOH proceeded differently from hydrogenation in neutral aqueous solution, it was of interest to investigate the hydrogenation of 4-amino-4-deoxy-PGA and of 2-deaminoPGA under similar conditions. When these compounds were hydrogenated under standard conditions in Warburg flasks the results shown in Fig. 2 were obtained. Unlike the hydrogenation of PGA, hydrogenation of 2-deamino-PGA proceeded at the same rate in neutral aqueous solution and in 0·1N-NaOH and was extremely slow after uptake of 1 mole/mole of 2-deaminoPGA.

In larger-scale experiments little more than 1 mole of H_2 was taken up even after prolonged vigorous shaking. In 0·1 N-NaOH 4-amino-4-deoxyPGA also readily took up only 1 mole of H_2 but at neutral pH H_2 uptake proceeded farther, though more slowly than with PGA.

Chromatography of hydrogenated PGA

With paper chromatography under conditions of low O_2 pressure it was found that when PGA was hydrogenated in the four solvents investigated the solution contained a number of blue-fluorescing compounds, as seen in Fig. 3. With the exception of the fluorescent spot of R_F 0·1–0·15, fluorescing areas were ill defined and generally displayed considerable 'tailing', but the general chromatographic pattern was consistently reproduced from one preparation to another if the experiments were performed under the same conditions.

The material produced when 1 mole of H_2/mole of PGA had been consumed appeared similar on chromatography to that produced in the same solvent when 2 moles of H_2 had been consumed (Fig. 3), except with 0·1 N-NaOH as solvent. When 1 mole of H_2/mole of PGA had been consumed, unreacted PGA was therefore probably still present in each case. Light-absorbing areas due to PGA were difficult to detect on the chromatograms, especially since fluorescent spots usually ran near PGA, but they could occasionally be seen, even when 2 moles of H_2/mole of PGA had been consumed.

With 0·1 N-NaOH as solvent for hydrogenation, the chromatogram produced by material after an uptake of 1 mole of H_2/mole of PGA showed only one main fluorescent spot. A minor fluorescent area of low R_F was due to an impurity present in the PGA. Because of the apparent purity of this preparation it has been used extensively in subsequent work. Hydrogenation in 0·1 N-NaOH to uptake of 2 moles of H_2/mole of PGA produced material giving chromatograms very similar to those produced at neutral pH.

Since paper chromatography under conditions of low O_2 pressure was not pursued with solvents other than 0·1 M-K_2HPO_4 to develop chromatograms, it is impossible to decide from the data presented whether the fluorescing compounds of similar R_F produced by hydrogenation in different solvents are identical or whether fluorescing compounds present before aeration are identical with fluorescing compounds present after aeration. The faintness and diffuseness of the fluorescing areas, the possibility of oxidation occurring even under conditions of low O_2 pressure and the large number of components present in the solutions, make it difficult to

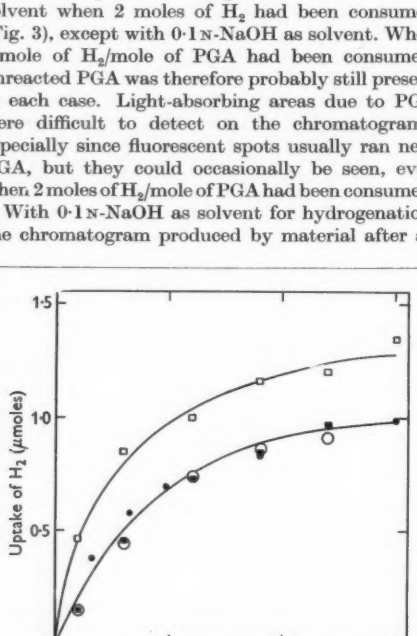


Fig. 2. Rate of hydrogenation of analogues of PGA in water. □, 4-Amino-4-deoxyPGA in neutral soln.; ○, 4-amino-4-deoxyPGA in 0·10 N-NaOH; ●, 2-deamino-PGA in neutral soln.; ■, 2-deaminoPGA in 0·1 N-NaOH. For conditions see text.

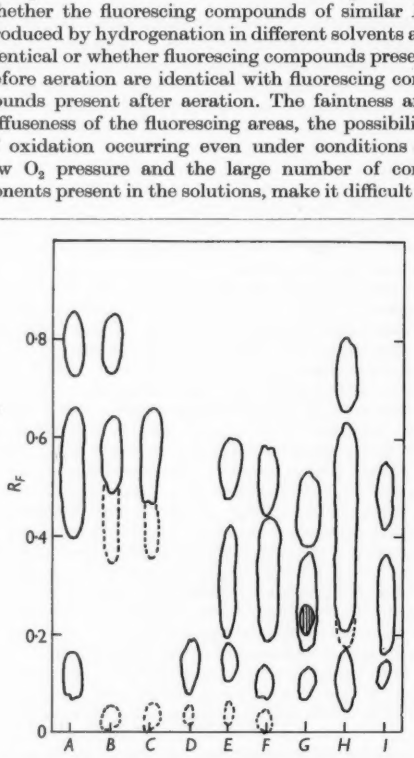


Fig. 3. Chromatograms of hydrogenated PGA. Outlined areas showed blue fluorescence and shaded area was dark over an ultraviolet lamp. Faintly fluorescent areas are enclosed by broken lines. The chromatogram was developed anaerobically with 0·1 M-K_2HPO_4. Material chromatographed and the solvent for hydrogenation were respectively as follows: *A* and *B*, dihydroPGA produced in formic acid; *C*, tetrahydroPGA in formic acid; *D*, dihydroPGA in 0·1 N-NaOH; *E*, tetrahydroPGA in 0·1 N-NaOH; *F*, dihydroPGA in water (neutral pH); *G*, tetrahydroPGA in water (neutral pH); *H*, dihydroPGA in acetic acid; *I*, tetrahydroPGA in acetic acid.

obtain significant results by such an approach. However, the data indicate that at least four distinct fluorescing compounds are formed either during the hydrogenation of PGA in various solvents or by an extremely rapid oxidation of tetrahydroPGA proceeding even at very low partial pressures of O_2.

Catalytic effect of PGA hydrogenated in different solvents

Hydrogenation of PGA in all four solvents, both when 1 and 2 moles of H_2/mole of PGA had been consumed, yielded products catalytically active in the synthesis of serine by the enzyme system. The test for coenzyme activity was, of course, most easily applied to PGA hydrogenated in neutral aqueous solution, since the solution of the product could be added directly, under N_2, to the enzyme system. Hydrogenated PGA produced in 0·1 N-NaOH was first neutralized by shaking under N_2+CO_2 (95:5, v/v) before testing. Solutions of hydrogenated products in acetic or formic acid were frozen on completion of hydrogenation and freeze-dried. The dry residue was dissolved in O_2-free water under N_2 and neutralized with solid $NaHCO_3$. Such preparations, though coenzyme active, gave much more erratic results than material produced in aqueous solution, for which accurate results are presented in a later paper of this series (Blakley, 1957). Because of the greater difficulty in handling products of hydrogenation of PGA in acetic or formic acid, further investigations have been confined to material produced in neutral aqueous solution or in 0·1 N-NaOH. In particular, PGA hydrogenated in 0·1 N-NaOH with uptake of 1 mole of H_2/mole of PGA (termed dihydroPGA) and PGA hydrogenated in neutral aqueous solution with uptake of 2 moles of H_2/mole of PGA (termed tetrahydroPGA) have been examined.

Rate of inactivation of hydrogenated PGA by aeration

Solutions of hydrogenated PGA produced in all four solvents quickly lost their coenzyme activity for serine synthesis when exposed to air. On the other hand, leucovorin (N^5-formyl-5:6:7:8-tetrahydroPGA) and anhydroleucovorin are stable to oxidation (Pohland, Flynn, Jones & Shive, 1951; Cosulich, Roth, Smith, Hultquist & Parker, 1951; May *et al.* 1951), the stability being attributed to the substituent groupings on N^5 of the pyrazine ring. A stable pteridine intermediate of serine synthesis from glycine and formaldehyde would probably also derive stability from substitution of N^5. Consequently, in seeking the stable intermediate it was important to be able to compare the oxidation rate of preparations under examination with the oxidation rate of dihydroPGA and tetrahydroPGA.

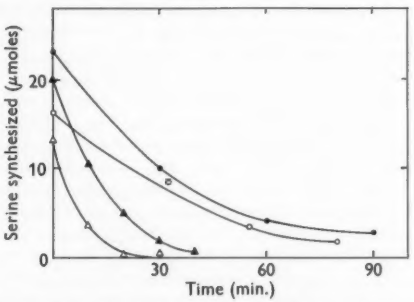


Fig. 4. Rate of loss of coenzyme activity by solutions of dihydroPGA and tetrahydroPGA during aeration in the presence and the absence of formaldehyde. Neutral aqueous solutions were vigorously shaken in air-filled test tubes. At intervals samples (0·45 ml.) of the solutions containing dihydroPGA or dihydroPGA + formaldehyde and samples (0·30 ml.) of the solutions containing tetrahydroPGA or tetrahydroPGA + formaldehyde were removed and tested with the serine-synthesizing enzyme system under standard conditions. △, 3·3 mM DihydroPGA; ○, 3·33 mM dihydroPGA + 5 mM formaldehyde; ▲, 3·33 mM tetrahydroPGA; ●, 3·33 mM tetrahydroPGA + 0·01 M formaldehyde.

Solutions of dihydroPGA and tetrahydroPGA were shaken in air-filled 6 in. × ½ in. test tubes on a Microid shaker at a rate sufficient to make the solution circulate over most of the surface of the test tube. Samples were removed at intervals and tested under identical conditions in the standard enzyme system. As may be seen from Fig. 4, solutions of tetrahydroPGA and dihydroPGA rapidly lost activity under these conditions and became completely inactivated in 30–40 min.

Nature of the inactivation of hydrogenated PGA during aeration

Previous workers have reported that hydrogenated PGA undergoes reoxidation to PGA on exposure to air (e.g. O'Dell, Vandenbelt, Bloom & Pfiffner, 1947), and although quantitative recovery was not obtained it has been assumed that hydrogenated PGA is rapidly oxidized in air by a simple reversal of the hydrogenation reaction (May *et al.* 1951).

To check this point the following experiment was executed. Duplicate preparations of dihydroPGA and of tetrahydroPGA (40 μmoles/1·5 ml.) were prepared in four Warburg flasks, aerated for 1 hr. in test tubes, and then allowed to stand for 9 days in the dark. Before and after aeration, and after 40 hr. and 9 days in the dark, samples were removed to estimate diazotizable aromatic amine, coenzyme activity for serine synthesis, H_2 uptake in the presence of platinum oxide catalyst and coenzyme

activity of the rehydrogenated material. The results are summarized in Tables 1 and 2.

Diazotizable aromatic amine could not be determined anaerobically because of oxidation of dihydroPGA or tetrahydroPGA during colour development. After aeration for 1 hr., however, there was a considerable amount of amine present and, assuming that this was *p*-aminobenzoylglutamic acid, about 16 μmoles of this material must have been formed from 40 μmoles of dihydroPGA and about 22 μmoles from 40 μmoles of tetrahydroPGA. Thus 40 % cleavage of dihydroPGA and 52 % cleavage of tetrahydroPGA appeared to occur during aeration. During standing for a further 9 days breakdown of the molecule progressed slowly with formation of 72 % of the theoretical yield of *p*-aminobenzoylglutamic acid from dihydroPGA and 78 % yield of tetrahydroPGA.

Most of the coenzyme activity of dihydroPGA and tetrahydroPGA was lost during aeration for 1 hr. When samples from these aerated solutions were again hydrogenated they consumed about 1·2 and 1·5 moles of H_2/mole of original PGA in the case of dihydroPGA and tetrahydroPGA respectively. During rehydrogenation these solutions appeared to regain about 90 % of their coenzyme activity. Hence, apart from the material from which *p*-aminobenzoylglutamic acid had been split, the remainder must have been converted back into the coenzyme.

On standing, less coenzyme activity could be restored to aerated solutions of dihydroPGA and tetrahydroPGA by rehydrogenation. After 9 days material from dihydroPGA gave only 13 % of the original coenzyme activity on rehydrogenation, while that from tetrahydroPGA gave only 35 % of the original activity on rehydrogenation.

Oxidative inactivation of dihydroPGA and tetrahydroPGA therefore appears to be due to two processes: (1) a rapid reaction, virtually completed during aeration for 1 hr., which produces a compound capable of rehydrogenation to coenzyme; (2) slower reaction(s) producing a diazotizable amine. The products of the latter reaction(s) do not yield coenzyme on rehydrogenation.

Chromatographic examination of solutions of oxidized dihydroPGA and tetrahydroPGA

Paper chromatography in air showed the presence of a number of fluorescent compounds in aerated solutions of dihydroPGA and tetrahydroPGA. Thus immediately after aeration, a chromatogram of material from tetrahydroPGA developed with 0·1 M-K_2HPO_4 as solvent showed four and probably five blue-fluorescing spots. The approximate R_F values for these fluorescent areas, together with other data, are given in Table 3. After allowing the aerated tetrahydroPGA to stand for 9 days, only three fluorescent compounds remained, of which one was impurity present in the PGA. Chromatography of this solution in several other solvents indicated that the major fluorescent compound was probably xanthopterin [2-amino-4:6-dihydroxypteridine, II (*a*)]; the presence of some PGA was also revealed. The weaker fluorescent spot was neither

Table 1. *Properties of material formed during oxidative breakdown of dihydroPGA*

Results are expressed in relation to an initial amount of 40 μmoles of dihydroPGA. Coenzyme activity was determined by adding material formed from 0·67 μmole of original PGA to the enzyme system under standard conditions.

Sample	Aromatic amine (as *p*-aminobenzoylglutamic acid) (μmoles)	H_2 consumed (mole/mole of original PGA)	Coenzyme activity (μmoles of serine/hr.)	Coenzyme activity after rehydrogenation (μmoles of serine/hr.)
DihydroPGA	.	.	13·6, 16·3	.
After aeration for 1 hr.	16, 15	1·32, 1·15	1·8, 1·8	13·6, 13·1
After 40 hr.	29, 22	1·56, 1·40	.	4·4, 5·4
After 9 days	31, 27	. , 0·88	.	3·8, 4·0

Table 2. *Properties of material formed during oxidative breakdown of tetrahydroPGA*

Results are expressed in relation to an initial amount of 40 μmoles of tetrahydroPGA. Coenzyme activity was determined by adding material formed from 0·67 μmole of original PGA to the enzyme system under standard conditions.

Sample	Aromatic amine (as *p*-aminobenzoylglutamic acid) (μmoles)	H_2 consumed (mole/mole of original PGA)	Coenzyme activity (μmoles of serine/hr.)	Coenzyme activity after rehydrogenation (μmoles of serine/hr.)
TetrahydroPGA	.	.	20·9, 20·5	.
After aeration for 1 hr.	22, 21	1·3, 1·6	5·9, 6·3	18·4, 16·4
After 40 hr.	23, 26	.	.	13·6, 12·0
After 9 days	32, 32	0·9, 0·8	.	8·0, 6·7

Table 3. *Fluorescent compounds from oxidized tetrahydroPGA and dihydroPGA appearing on chromatograms*

Chromatograms were developed with 0·1 M-K_2HPO_4.

R_F of fluorescent compound	Intensity of fluorescence: After aeration for 1 hr.	After 40 hr.	After 9 days	Remarks
TetrahydroPGA				
0·64	+ +	+ +	.	Identity unknown
0·52	+ +	+ +	+ + +	Probably xanthopterin
0·42	+ +	+ + +	+ +	Identity unknown
0·31	+ + +	+ +	.	When paper is kept, spot turns yellow and non-fluorescent
0·03–0·10	+	+	+	Probably impurity from PGA
DihydroPGA				
0·52–0·58	+ +	+ + +	+ +	Probably xanthopterin
0·38	+	.	.	Identity unknown
0·20–0·23	+ + +	+	.	When paper is kept, spot turns yellow and non-fluorescent
0·12	.	+ + +	+ +	Identity unknown
0·03–0·10	+	+	.	Probably impurity from PGA

2-amino-4-hydroxy-6-methylpteridine [II (*b*)] nor 2-amino-4-hydroxypteridine-6-carboxylic acid [II (*c*)], and its identity is unknown.

As in the case of oxidized tetrahydroPGA solutions, a chromatogram of recently aerated dihydroPGA revealed a number of fluorescent compounds (Table 3), but after the aerated solution had been allowed to stand a chromatogram showed only one of these compounds and one new, intensely fluorescent compound. The identity of these fluorescent compounds is also unknown except for the spot tentatively identified with xanthopterin.

The effect of light and pH of the solution on the course of oxidation of dihydroPGA and tetrahydroPGA has also been investigated by means of paper chromatography. Neither was found to influence appreciably the number or intensity of the fluorescent spots appearing on the chromatograms.

Isolation of PGA, xanthopterin and 2-amino-4-hydroxy-6-methylpteridine from oxidation products of hydrogenated PGA

Chromatography on a Solka Flok column enabled separation in an almost pure state of several components from the oxidation products of hydrogenated PGA. PGA was hydrogenated in formic acid (1 mole of H_2/mole of PGA), the solvent removed by freeze-drying and the residue dissolved in water and neutralized. The coenzyme-active solution oxidized on exposure to air to give one major fluorescent product which could be eluted as a single relatively pure band from the Solka Flok column with 5% (v/v) acetic acid, appearing in tubes 80–100 (20 ml. fractions). The eluate containing this band was freeze-dried and the residue extracted with 0·1 N-NaOH. The absorption spectrum of the centrifuged extract was determined at pH 1, 7·2 and 13, and was found to show a close resemblance to that of 2-amino-4-hydroxy-6-methylpteridine (Fig. 5). Paper chromatography in a number of solvents confirmed the identity of the fluorescent material as the 6-methyl derivative, since the two compounds had similar R_F values in the following solvent systems: *sec.*-butanol–formic acid–water (R_F 0·89); *n*-butanol–acetic acid–water (R_F 0·51); propanol–NH_3 soln. (R_F 0·64); 0·1 M-K_2HPO_4 (R_F 0·49); 5% (v/v) acetic acid (R_F 0·68); 0·1 N-Na_2CO_3 (R_F 0·57); 3% (w/v) NH_4Cl (R_F 0·51).

It was possible to isolate three compounds in major amounts from oxidized tetrahydroPGA or dihydroPGA which had been prepared in neutral or alkaline aqueous solution respectively. The first eluted from the Solka Flok column by 9·6% (w/v) ammonium carbonate solution (tubes 50–60, 20 ml. fractions) was a diazotizable amine similar in absorption spectrum ($\lambda_{max.}$ 273 mμ.) to *p*-aminobenzoylglutamic acid. Paper chromatography in propanol–NH_3 soln., in *n*-butanol–acetic acid–water, and in *n*-butanol saturated with water confirmed its identity as *p*-aminobenzoylglutamic acid and distinguished it from *p*-aminobenzoic acid. Oxidation products from dihydroPGA or tetrahydroPGA did, however, sometimes contain appreciable amounts of *p*-aminobenzoic acid in addition to *p*-aminobenzoylglutamic acid.

Fractions from the column which contained the other two compounds usually overlapped (tubes 85–110, 20 ml. fractions), but after freeze-drying the fractions containing them and dissolving the residue, they could be separated by difference in solubility at acid pH. The ultraviolet-light-absorbing compound coming off the column slightly earlier was almost quantitatively precipitated at pH 3. A solution of the precipitate gave absorption

spectra identical with those of PGA at pH 1, 7·3 and 13. Comparison of the material with PGA by paper chromatography showed that the compounds had identical R_F values in the following solvent systems: 3% (v/v) NH_4Cl (R_F 0·25); 0·1 M sodium acetate buffer, pH 5·0 (R_F 0·40); propanol–NH_3 soln. (R_F 0·20); 0·1 M-K_2HPO_4 (R_F 0·35); 0·1 N-Na_2CO_3 (R_F 0·62).

Absorption spectra of the fluorescent compound remaining in the solution at acid pH were similar to those of xanthopterin at pH 1, 5 and 13, as seen in Fig. 6. Moreover, paper chromatography with n

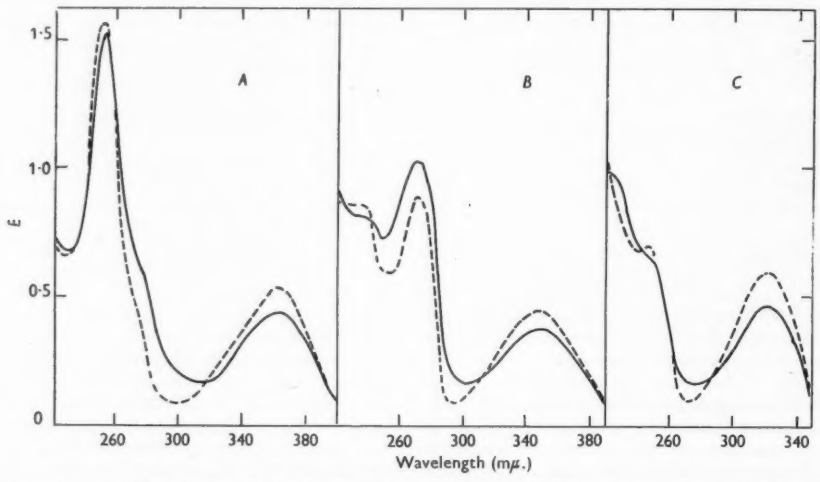


Fig. 5. Comparison of absorption spectra of 2-amino-4-hydroxy-6-methylpteridine and material isolated from oxidized tetrahydroPGA. ——, Isolated material; – – –, 2-amino-4-hydroxy-6-methylpteridine. *A*, In 0·1 N-NaOH; *B*, in 0·1 M potassium phosphate buffer, pH 7·3; *C*, in 0·1 N-HCl.

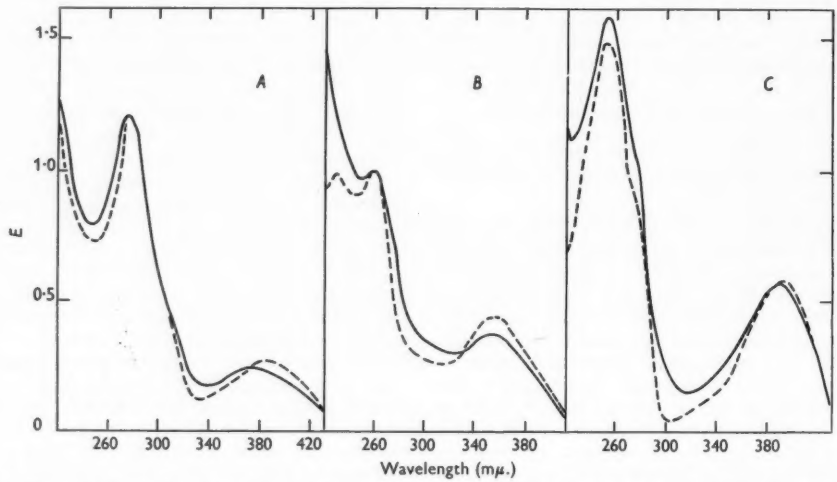


Fig. 6. Comparison of absorption spectra of xanthopterin and material isolated from oxidized tetrahydroPGA. ——, Isolated material; – – –, xanthopterin. *A*, In 0·1 M sodium acetate buffer, pH 5·0; *B*, in 0·1 N-HCl; *C*, in 0·1 N-NaOH.

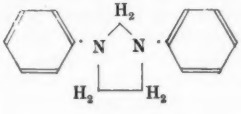
number of solvents showed the material to have the same R_F as xanthopterin in all cases. The following solvent systems were used: *sec.*-butanol–formic acid–water (R_F 0·58); *n*-butanol–acetic acid–water (R_F 0·52); 3 % (w/v) NH_4Cl (R_F 0·44); 0·1 N-Na_2CO_3 (R_F 0·65); 5 % (w/v) boric acid (R_F 0·54); propanol–NH_3 soln. (R_F 0·35); 5 % (v/v) acetic acid (R_F 0·56); 0·1 M-K_2HPO_4 (R_F 0·51).

Quantitative aspects of the breakdown of hydrogenated PGA

Recovery of pteridines of known structure from the oxidation products of dihydro- and tetrahydro-PGA accounted for only a small proportion of the original PGA, as may be seen from Table 4, which gives representative results. The reason for such low recoveries of pteridines is not known, but part of the loss occurred during the chromatography, concentration and handling of the pteridine solutions. The components of a synthetic mixture of *p*-aminobenzoylglutamic acid, xanthopterin and PGA, when chromatographed on the Solka Flok column, collected and prepared by the usual technique, were recovered in 70–90 % yield. This suggests that the low recoveries of PGA and xanthopterin from tetrahydroPGA were mainly due to the conversion of most of the tetrahydroPGA into products so far undetected.

Stability of compounds formed between formaldehyde and hydrogenated PGA

Formaldehyde reacts very readily with 1:2-diamines to form stable cyclic compounds. Thus *NN'*-diphenylethylenediamine reacted readily with formaldehyde to form a stable compound (III) (see Methods) from which free formaldehyde could not be recovered by steam-distillation from a neutral, strongly acid, or alkaline solution. The absorption spectrum of this compound was found to have two maxima (254 and 295 mμ.; $\epsilon_{max.}$ $3{\cdot}94 \times 10^4$ and $5{\cdot}4 \times 10^3$ respectively) and is thus very similar to that of the parent *NN'*-diphenylethylenediamine (maxima at 250 and 294 mμ.; $\epsilon_{max.}$ $2{\cdot}76 \times 10^4$ and 5×10^3 respectively).



(III)

Attempts to detect a similar stable compound in which formaldehyde is condensed across the N^5 and N^{10} positions of tetrahydroPGA furnished no evidence for the non-enzymic formation of such a substance. After incubating tetrahydroPGA or dihydroPGA with formaldehyde (at various concentrations), paper chromatography never revealed the presence of components other than those already present in the hydrogenated PGA or formed from it during chromatography.

Further evidence against the formation of a stable condensation product of formaldehyde and hydrogenated PGA was obtained by studies on the stability to aeration of hydrogenated PGA treated with formaldehyde. Solutions of dihydroPGA or tetrahydroPGA lost most of their coenzyme activity during a 30 min. aeration period (cf. Fig. 4), but during aeration tetrahydroPGA or dihydroPGA treated with varying amounts of formaldehyde retained much more coenzyme activity for serine synthesis. The activity remaining after aeration was found to depend on the molar ratio of formaldehyde: hydrogenated PGA. As may be seen from Table 5, after aeration for 30 min., tetrahydroPGA which had been treated with about three molecular proportions of formaldehyde retained more coenzyme activity for serine synthesis than tetrahydroPGA treated with either a larger or a smaller proportion

Table 4. *Recovery of* p*-aminobenzoylglutamic acid, xanthopterin and pteroylglutamic acid from the oxidation products of dihydroPGA and tetrahydroPGA*

The yields are those obtained from 100 μmoles of dihydroPGA or of tetrahydroPGA.

Product	From tetrahydroPGA (μmoles)	From dihydroPGA (μmoles)
p-Aminobenzoylglutamic acid	56	51
Xanthopterin	17	5
PGA	12	4

Table 5. *Effect of the addition of various amounts of formaldehyde in preserving coenzyme activity of tetrahydroPGA during aeration*

Varying amounts of formaldehyde were added under N_2 to each of four Warburg vessels each containing 10 μmoles of tetrahydroPGA in 3·0 ml. of water, pH 7. After 30 min. at 37° under N_2 the solutions were transferred to test tubes and shaken vigorously on a Microid shaker under air at room temp. for 30 min. From each tube 0·3 ml. samples were withdrawn and tested under standard conditions with the serine-synthesizing enzyme.

	Formaldehyde added (μmoles/μmole of tetrahydroPGA)	Serine synthesized (μmoles)
Expt. 1	0	6·1
	1·0	12·0
	3·0	20·5
	6·0	16·5
Expt. 2	1·0	7·5
	2·0	10·3
	3·0	15·3
	4·0	13·0

Table 6. *Effect of the addition of various amounts of formaldehyde in preserving coenzyme activity of dihydroPGA during aeration*

Varying amounts of formaldehyde were added under N_2 to each of four Warburg vessels each containing 10 μmoles of dihydroPGA in 3·0 ml. of water, pH 7. The solutions after 30 min. under N_2+CO_2 (95:5, v/v) at 37° were transferred to test tubes and shaken vigorously on a Microid shaker under air for 30 min. From each tube 0·45 ml. samples were then withdrawn and tested under standard conditions with the serine-synthesizing enzyme.

	Formaldehyde added (μmoles/μmole of dihydroPGA)	Serine synthesized (μmoles)
Expt. 1	0·5	8·2
	1·0	11·4
	1·5	14·2
Expt. 2	1·5	12·7
	3·0	11·8
	4·5	9·9

of formaldehyde, and retained 2–3 times as much activity as aerated tetrahydroPGA to which no formaldehyde was added. Table 6 shows that addition of about 1·5 molecular proportions of formaldehyde was most effective in stabilizing dihydroPGA against loss of coenzyme activity during aeration.

To obtain a better indication of the stability of the formaldehyde-treated dihydroPGA and tetrahydroPGA, their coenzyme activity was determined after various periods of aeration under the same conditions employed to obtain the results for dihydroPGA and tetrahydroPGA in the absence of formaldehyde (Fig. 4). As may be seen in Fig. 4, the coenzyme activity of the formaldehyde-treated compounds was still considerable after aeration for 40 min., at which time the untreated compounds had lost practically all their activity. However, even the formaldehyde-treated materials had lost 95 % of their activity after aeration for 1·5 hr.

DISCUSSION

When PGA was hydrogenated in various solvents and the products were chromatographed under conditions of low oxygen tension, the chromatograms showed under ultraviolet light a number of blue-fluorescing areas. The nature of these fluorescent derivatives of PGA is unknown, and it is uncertain whether they are actually present in the solution of hydrogenated PGA or, as seems more likely, formed very rapidly in the presence of oxygen. Even if they are secondary products the variation of their nature and number with the solvent employed for hydrogenation indicates that the primary products of the hydrogenation of PGA must also vary with the solvent.

When water is used as solvent the course of hydrogenation at pH 5–9 is different from that at pH 13. In the latter case hydrogenation proceeds rapidly until an uptake of 1 mole of hydrogen/mole of PGA is reached. A further mole of hydrogen reacts only with difficulty. Chromatograms of the dihydroPGA produced in 0·1 N-NaOH show only one brightly fluorescent area. Below pH 9 aqueous solutions of PGA rapidly take up 2 moles of hydrogen/mole of PGA and the product exhibits three or more fluorescent areas on paper chromatograms.

Since the most important change in the PGA molecule produced by raising the pH from 7 to 13 is the ionization of the 4-hydroxyl group, it might be surmised that the ionization of this grouping profoundly influences the course of hydrogenation. It is generally assumed that the pyrazine ring rather than the pyrimidine ring of PGA becomes hydrogenated, giving 5:6:7:8-tetrahydroPGA, since pyrazine is hydrogenated under milder conditions than are pyrimidines. The marked effect of the ionization of the hydroxyl group on the pyrimidine ring would therefore be surprising. For this reason the hydrogenation of 4-amino-4-deoxyPGA and of 2-deaminoPGA was also investigated. The behaviour of the former compound was similar to that of PGA despite their difference in structure, although hydrogenation in neutral aqueous solution was considerably slower than with PGA under these conditions. On the other hand, hydrogenation of 2-deaminoPGA proceeded at the same rate in neutral aqueous solution as in 0·1 N-NaOH, only 1 mole of hydrogen being readily taken up/mole of 2-deaminoPGA. The results are difficult to interpret but suggest that substituents of the pyrimidine ring influence the course of hydrogenation.

All preparations of both dihydroPGA and tetrahydroPGA were active as coenzyme in enzymic serine synthesis. It has been postulated that the structure of partially hydrogenated PGA is 7:8-dihydroPGA (O'Dell *et al.* 1947). If this is the case the N^8 position of the pteridine ring is presumably capable of forming a reactive grouping with formaldehyde as well as the N^5 position, and in 5:6:7:8-tetrahydroPGA two reactive groupings at N^5 and N^8 might be formed. It has been generally assumed that the N^5 position of the pteridine ring is that concerned with coenzyme activity because of the N^5-formyl grouping in leucovorin, but this evidence alone does not exclude the participation of the N^8 position in the reaction also.

Oxidation and degradation of hydrogenated PGA

As reported by other workers (O'Dell *et al.* 1947), dihydroPGA and tetrahydroPGA are rapidly oxidized in air, and in the present work it has been

found that the product is inactive in the serine-synthesizing enzyme system. Chromatography revealed that only a small amount of PGA could be recovered from the products of oxidation and that there was considerable splitting off from the molecule of *p*-aminobenzoylglutamic acid with simultaneous formation of fluorescent pteridines. This was confirmed by determination of diazotizable amine formed during oxidation. Xanthopterin was identified among the products, a fact which raises the interesting question of the *in vivo* relation between this compound and PGA.

Immediately after solutions of dihydroPGA or tetrahydroPGA had been inactivated by aeration a substantial portion of their coenzyme activity could be recovered by rehydrogenation, yet paper chromatograms showed that little PGA was present although several unidentified brightly fluorescing compounds could be seen. Hence the initial product of the oxidation of dihydroPGA or tetrahydroPGA in air appears to be one or more fluorescing compounds of unknown structure which subsequently lose their *p*-aminobenzoylglutamic acid side chain with formation of simpler pteridines such as xanthopterin.

Treatment of dihydroPGA and tetrahydroPGA with formaldehyde results in a decreased loss of coenzyme activity during aeration, but no evidence was obtained for the reaction of formaldehyde with hydrogenated PGA to form a compound with a stability to mild oxidation comparable with that of leucovorin. The fact that formaldehyde did afford dihydroPGA and tetrahydroPGA some protection from oxidation indicates a combination of formaldehyde with PGA, probably with N^5- and N^8-amine groups. The fact that the maximum protection was afforded by about 3 moles of formaldehyde/mole of tetrahydroPGA and 1·5 moles of formaldehyde/mole of dihydroPGA, suggests that for serine synthesis formaldehyde combines with only one grouping of dihydroPGA and two of tetrahydroPGA. Since the products of this combination were relatively unstable to oxidation, formaldehyde is probably involved in a freely dissociable combination such as the formation of a hydroxymethyl group.

The maintenance of coenzyme-active forms of PGA in living tissue is difficult to explain in view of the great instability of hydrogenated PGA *in vitro*, but it may depend on the effects of the combination of tetrahydroPGA with the native enzyme or on almost complete combination *in vivo* of the hydrogenated PGA with formaldehyde or other one-carbon molecule.

SUMMARY

1. Pteroylglutamic acid (PGA), when hydrogenated in the presence of platinum oxide, takes up two molecules of hydrogen in formic acid, acetic acid or neutral aqueous solution. In dilute alkali only one mole of hydrogen reacts rapidly but a further mole is taken up on prolonged shaking. Hydrogenation in formic acid or neutral aqueous solution is more rapid than in the other solvents.

2. Paper chromatograms of the hydrogenation products developed with 0·1 M-K_2HPO_4 in an atmosphere of nitrogen show a number of fluorescing spots. The number and the R_F values of these spots depend on the solvent employed for the hydrogenation. DihydroPGA formed in dilute alkali showed only one main fluorescent spot on chromatography.

3. DihydroPGA and tetrahydroPGA preparations were all active in stimulating serine synthesis by preparations of rabbit-liver enzyme, irrespective of the solvent employed for hydrogenation.

4. Solutions of dihydroPGA and tetrahydroPGA lost their coenzyme activity for serine synthesis in 30–40 min. when vigorously shaken in air. This activity could only partly be stored by rehydrogenation, and evidence was obtained for a considerable loss of the *p*-aminobenzoylglutamic acid side chain, especially when the solutions had been allowed to stand some days.

5. A number of brightly fluorescing compounds, probably simple pteridines, are ultimately formed by oxidative degradation of hydrogenated PGA in air. Among these, xanthopterin and 2-amino-4-hydroxy-6-methylpteridine have been identified. PGA accounts for only a small fraction of the oxidation products.

6. No new stable pteridine could be detected by paper chromatography in solutions containing formaldehyde and dihydroPGA or tetrahydroPGA.

7. Incubation of dihydroPGA or tetrahydroPGA with formaldehyde affords slight protection against loss of coenzyme activity during aeration but does not result in a coenzyme appreciably stable to oxidation.

8. DihydroPGA retained most coenzyme activity during aeration when treated with 1·5 molecular proportions of formaldehyde, and tetrahydroPGA retained most when treated with three molecular proportions.

9. This evidence suggests that dihydroPGA forms mono(hydroxymethyl)dihydroPGA as an intermediate in serine synthesis whereas tetrahydroPGA forms bis(hydroxymethyl)tetrahydroPGA.

My thanks are due to those who generously supplied reference compounds as acknowledged above, and to Dr A. L. Morrison of Roche Products Ltd. for a further gift of crystalline pyridoxal phosphate. I am also indebted to Professor A. H. Ennor for his constant interest and advice.

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The Interconversion of Serine and Glycine: some Further Properties of the Enzyme System

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The partial purification from rabbit liver of a soluble enzyme system which catalyses the net synthesis of serine from glycine and formaldehyde has been previously reported (Blakley, 1955), together with evidence for the participation of pyridoxal phosphate. Further work has had as its chief aim elucidation of the role of tetrahydropteroylglutamic acid (tetrahydroPGA) in this enzymic reaction, but in the course of these investigations certain properties of the enzyme system have been determined. It is the purpose of this paper to present these results, the most important of which concern the specificity of the enzyme system for tetrahydroPGA as the coenzyme.

EXPERIMENTAL

Materials

Pteridines. Commercial samples of pteroylglutamic acid (PGA) were purified as previously described (Blakley, 1957). N^{10}-FormylPGA and calcium leucovorin (calcium N^5-formyl-5:6:7:8-tetrahydropteroylglutamate) were gifts from Dr H. P. Broquist of Lederle Laboratories. Pteroic acid, 4-amino-4-deoxyPGA (aminopterin), sodium pteroyltriglutamate (teropterin) and anhydroleucovorin A were gifts from Dr T. H. Jukes of Lederle Laboratories. 2-DeaminoPGA was a gift from Professor A. Albert and Dr D. J. Brown of the Department of Medical Chemistry of this University. Citrovorum factor was kindly supplied as the crystalline barium salt by Drs Silverman and Keresztesy of the National Institutes of Health, Bethesda, U.S.A. Pteroylheptaglutamic acid was a gift from Dr J. J. Pfiffner of Parke, Davis and Co., Detroit, Michigan, U.S.A., obtained through the courtesy of Dr H. P. Broquist. The 5:6:7:8-tetrahydro derivatives of PGA, N^{10}-formylPGA, 4-amino-4-deoxyPGA, pteroyltriglutamic acid and pteroylheptaglutamic acid and dihydro-2-deaminoPGA were prepared by catalytic hydrogenation in neutral aqueous solution as previously described (Blakley, 1957). DihydroPGA was prepared by hydrogenation in 0·1 N-NaOH (Blakley, 1957), the solution being neutralized before use by shaking under $N_2 + CO_2$ (95:5, v/v).

DL-[3-^{14}C]Serine was obtained from the Radiochemical Centre, Amersham, and contained 0·1 mc in 11·3 mg.

Pyridoxal phosphate monohydrate. A generous gift of the crystalline compound was received from Dr A. L. Morrison of Roche Products Ltd., Welwyn Garden City, England.

METHODS

Enzyme. This was prepared as previously described (Blakley, 1955). All experiments were performed with enzyme purified by the complete procedure and having specific activity 12–25 μmoles of serine/hr./mg. of protein.

Determination of enzyme activity. The manometric method, depending on serine estimation by oxidation with periodate, was used as reported earlier (Blakley, 1955). The standard enzyme system consisted of enzyme (to give 10–20 μmoles of serine/hr. under optimum conditions), 0·01 M-$NaHCO_3$, 5×10^{-4} M pyridoxal phosphate, 1·3 mM tetrahydroPGA and water to bring the final volume to 3·0 ml. Glycine and formaldehyde were added from the side arm after equilibrating the solution at 37° under $N_2 + CO_2$ (95:5, v/v) so that their final concentrations were glycine, 0·1 M and formaldehyde, 0·01 M. Any departure from these conditions is indicated in the legends to tables and figures.

RESULTS

Proportionality of serine formed to reaction time and amount of enzyme

With the standard-assay system and about 0·5 mg. of enzyme the rate of serine synthesis proceeded uniformly for a period of more than an hour (Fig. 1).

Under the standard conditions serine synthesis, in a period of 1 hr., was found to be proportional to the amount of enzyme added within a wide range of enzyme concentrations (Fig. 2). These results were the basis for regarding serine synthesis during a period of 1 hr. as a measure of enzyme activity in this and previous work (Blakley, 1955).

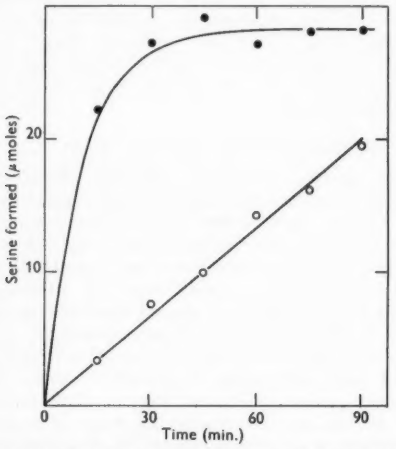

Fig. 1. Effect of period of incubation on serine synthesis. The standard enzyme system was incubated for various periods of time before heat-inactivation of the enzyme and determination of serine; ○, 0·36 mg. of protein/flask, ●, 1·8 mg. of protein/flask.

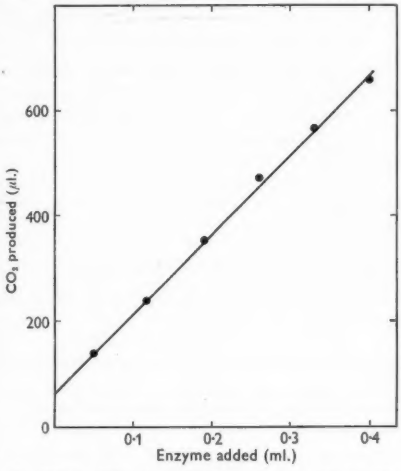

Fig. 2. Effect of the amount of enzyme on serine synthesis. Serine synthesis was measured manometrically as CO_2 after periodate oxidation, as described in the text.

With an excess of enzyme the reaction reached an equilibrium corresponding to the formation of about 28·2 μmoles of serine (Fig. 1). This result enables an approximate estimation of the equilibrium constant for the overall reaction:

$$HO.CH_2.CH(NH_2).CO_2H \rightleftharpoons H_2N.CH_2.CO_2H + H.CHO$$

defined by

$$K = [\text{glycine}]\,[\text{H.CHO}]/[\text{serine}]$$

of value 5·8 mM.

More accurate determination of the equilibrium constant by studying the reaction in the reverse direction has not been accomplished because of analytical problems.

Effect of substrate concentration on reaction rates

The effect of glycine concentration on reaction velocity is shown in Fig. 3, where the results are plotted according to the method of Lineweaver & Burk (1934). It may be seen that they conform to

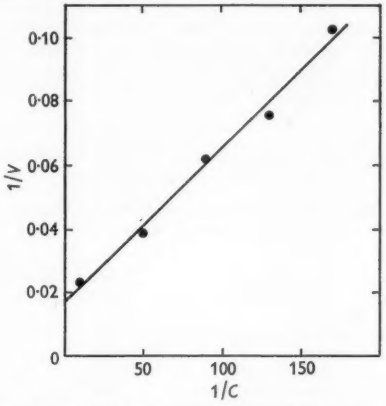

Fig. 3. Effect of glycine concentration on serine synthesis. The results are plotted according to the method of Lineweaver & Burk (1934). *C*, Molar concentration of glycine; *V*, μmoles of serine formed under standard conditions in 1 hr.

Table 1. *Effect of formaldehyde concentration on rate of serine synthesis*

The reaction was performed under standard conditions, except that the concentration of formaldehyde was varied as shown.

Formaldehyde concn. (M)	Serine synthesized (μmoles/hr.)
0·0025	4·6
0·0050	11·9
0·0100	22·1
0·0150	17·3
0·0200	15·6
0·0250	12·6

the requirements of a simple Michaelis–Menten relation over the concentrations used, giving an apparent Michaelis constant with the unusually high value of $2{\cdot}8 \times 10^{-2}$.

As may be seen from Table 1, the rate of serine synthesis increased with formaldehyde concentration up to 0·01 M, but further increase of formaldehyde concentration diminished the synthesis of serine.

Table 2. *Substrate specificity of the enzyme system for serine synthesis*

The reaction was performed under standard conditions (see text), except that the substrates were 0·10 M glycine and those shown below.

Substrate	β-Hydroxy-α-amino acid synthesized (μmoles/hr.)
Expt. 1	
Formaldehyde (0·01 M)	12·8
Acetaldehyde (0·01 M)	0·2
Sodium formate (0·01 M)	0·0
Expt. 2	
Formaldehyde (0·01 M)	23·7
Acetaldehyde (0·025 M)	0
Formaldehyde (0·01 M) +acetaldehyde (0·025 M)	22·8

Table 3. *Activating effect on serine synthesis of pteridines related to PGA*

Pteridines were tested at the concentration shown in the standard enzyme system with an excess of enzyme present.

Expt. no.	Pteridine	Concn. (mM)	Serine synthesized (μmoles/hr.)
1	None	—	0
	Citrovorum factor	1·88	0
	Leucovorin	1·88	0
	2-DeaminoPGA	1·88	0·1
	4-Deoxy-4-aminoPGA	1·88	0
	Pteroyltriglutamic acid	1·88	0
	N^{10}-FormylPGA	1·88	1·1
	TetrahydroPGA	1·88	12·0
2	None	—	0
	Tetrahydro-2-deaminoPGA	2·50	0
	Anhydroleucovorin A	2·50	0
	PGA	2·50	0
	Tetrahydro-4-amino-4-deoxyPGA	2·50	0
	TetrahydroPGA	2·50	12·5
3	None	—	0
	TetrahydroPGA	0·33	16·2
	Tetrahydro-N^{10}-formylPGA	0·33	5·0
	Tetrahydropteroyltri-glutamic acid	0·33	11·5
4	None	—	0
	TetrahydroPGA	0·33	8·6
	Pteroic acid	0·33	0·2
5	None	—	0
	Pteroylheptaglutamic acid	0·093	3·4
	Pteroylheptaglutamic acid	0·038	2·9
	TetrahydroPGA	0·33	24·1

Substrate specificity

Acetaldehyde and formate were unable to replace formaldehyde in the reaction (Table 2). The volatility of acetaldehyde introduces some uncertainty as far as its concentration in solution is concerned, but its lack of activity is in accord with previous reports (Vilenkina, 1952; Blakley, 1954). When added at a concentration of 0·025 M, acetaldehyde did not produce any inhibition of serine synthesis from glycine and formaldehyde.

No serine synthesis occurred when sarcosine (0·1 M) was used in place of glycine in the purified enzyme system.

Specificity of the enzyme for tetrahydroPGA

As reported previously (Blakley, 1957), the enzyme is activated both by dihydroPGA and tetrahydroPGA. Since several other related pteridines have been suggested as possessing coenzyme properties a number of these have been tested with the rabbit-liver enzyme system. The only substances tested which consistently caused significant activation of the enzyme were tetrahydroPGA (and dihydroPGA), tetrahydro-N^{10}-formylPGA, and the tetrahydro derivatives of pteroyltriglutamic and pteroylheptaglutamic acids (Table 3). Consequently the enzymic synthesis of serine does not involve formation of anhydroleucovorin A, leucovorin or the citrovorum factor, and only hydrogenated derivatives of PGA are active. The activity of the hydrogenated PGA derivatives containing several glutamic acid residues in the molecule is noteworthy.

Michaelis constants for dihydroPGA, tetrahydroPGA and tetrahydropteroyltriglutamic acid

When rigid precautions were taken to prevent their reoxidation during the experiment, dihydroPGA, tetrahydroPGA and tetrahydropteroyltriglutamic acid gave results conforming to the simple Michaelis–Menten relation as seen in Figs. 4 and 5. The values for the Michaelis constants were approximately $2{\cdot}9 \times 10^{-4}$ M, $1{\cdot}8 \times 10^{-4}$ M and $8{\cdot}3 \times 10^{-5}$ M respectively.

Inhibition of serine synthesis by analogues of PGA

It is well known that 4-amino-4-deoxyPGA and 2-deaminoPGA are antagonists of PGA *in vivo* (Jukes, 1953; Nimmo-Smith & Brown, 1953). The hydrogenated derivatives of these compounds were therefore tested as inhibitors for the enzymic synthesis of serine, with the results shown in Table 4. In accordance with previous results (Blakley, 1954) tetrahydro-4-amino-4-deoxyPGA did not inhibit the action of tetrahydroPGA in the enzyme reactions, the antagonism of 4-amino-4-deoxyPGA to PGA *in vivo* being due rather to inhibition of the

enzyme system responsible for reducing PGA to its hydrogenated derivative. Dihydro-2-deaminoPGA produced significant inhibition of the synthesis of serine, however, and preliminary results from data plotted according to the method of Lineweaver & Burk (1934) indicate that the inhibition is competitive.

Pteridine intermediate in serine synthesis

The role of tetrahydroPGA in serine synthesis presumably lies in the activation of formaldehyde. Evidence has been presented to indicate that no stable compound is formed by non-enzymic combination of formaldehyde and tetrahydroPGA, although it is possible that readily dissociating compounds, such as those containing hydroxymethyl groups, may be formed (Blakley, 1957). It is conceivable, however, that in the presence of a component of the enzyme system, formaldehyde and tetrahydroPGA could combine to form a long-lived intermediate, stable to oxidation.

Two methods have been used to assist the possible accumulation of such an intermediate: (*a*) incubation of enzyme, formaldehyde (30 μmoles) and tetrahydroPGA (10 μmoles) under N_2, and (*b*) incubation of enzyme, serine (0·1–0·4 M) and tetrahydroPGA (10 μmoles) under N_2. In both cases the amount of enzyme and the period of incubation chosen were such that in previous tests with the complete serine-synthesizing system they resulted in the production of 20 μmoles of serine. When method (*a*) was used the enzyme was inactivated after incubation by heating the mixture under N_2 in a boiling-water bath for 2 min. In method (*b*), after incubation the enzyme was precipitated by 3 vol. of ethanol (99 %, v/v) and the pteridines were precipitated from the supernatant by the addition of 40 μmoles of barium acetate and another 1 vol. of ethanol. The flocculent precipitate was collected

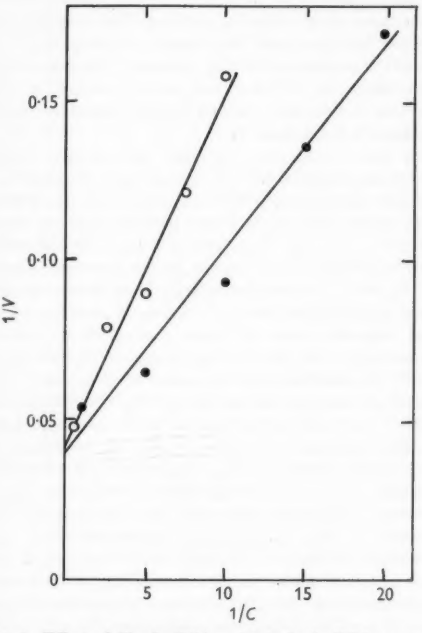

Fig. 4. Effect of dihydroPGA and tetrahydroPGA concentration on serine synthesis. The results are plotted according to the method of Lineweaver & Burk (1934). *C*, 900 × Pteridine concentration (M); *V*, μmoles of serine formed under standard conditions in 30 min.; ○, with dihydroPGA; ●, with tetrahydroPGA.

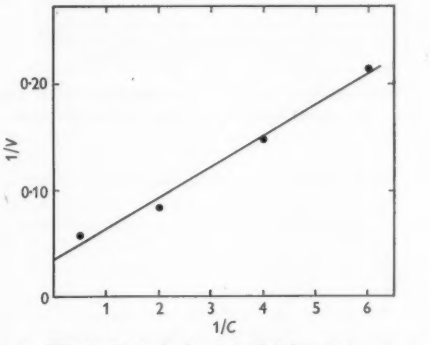

Fig. 5. Effect of tetrahydropteroyltriglutamate concentration on serine synthesis. The results are plotted according to the method of Lineweaver & Burk (1934). *C*, Tetrahydropteroyltriglutamate concentration (10^{-4} M); *V*, μmoles of serine formed under standard conditions in 1 hr.

Table 4. *Effect of tetrahydro-4-amino-4-deoxyPGA and dihydro-2-deaminoPGA on serine synthesis*

Standard conditions were used except that the concentration of tetrahydroPGA was $2·5 \times 10^{-4}$ M.

Inhibitor	Concn.	Serine synthesized (μmoles/hr.)	Inhibition (%)
None	—	14·8	—
Tetrahydro-4-amino-4-deoxyPGA	$7·5 \times 10^{-4}$ M	15·1	0
Dihydro-2-deaminoPGA	$7·5 \times 10^{-4}$ M	6·6	55

by centrifuging under N_2 in the cold, washed with ethanol and dried *in vacuo*.

The reaction medium from method (*a*) and solutions of the barium salt from method (*b*) were examined by paper chromatography for evidence of a new pteridine component. The detection of the postulated intermediate on chromatograms was complicated by the presence of numerous fluorescent compounds formed by oxidative degradation of tetrahydroPGA (Blakley, 1957), and no new compound could be found. Separation of some of the components of the barium salt from method (*b*) by chromatography on Solka Flok (powdered cellulose) columns after removal of Ba^{2+} ions yielded no coenzyme-active compounds which were stable to oxidation. Although some compounds obtained by chromatography on Solka Flok showed a slight coenzyme activity as isolated and considerable coenzyme activity on rehydrogenation, the same was true of solutions of oxidized tetrahydroPGA (Blakley, 1957).

To check this point further the coenzyme activity of a solution obtained by method (*a*) was tested after vigorous shaking in air for various periods. The results, shown in Fig. 6, do not differ appreciably from those obtained with a solution of tetrahydroPGA and formaldehyde (Blakley, 1957).

Effect of incubating [3-^{14}C]*serine with enzyme and tetrahydroPGA*

As a further test for the formation of a stable pteridine intermediate in serine–glycine interconversion, [3-^{14}C]serine has been incubated with enzyme and tetrahydroPGA and the pteridines isolated, chromatographed and examined for radioactivity. DL-[3-^{14}C]Serine (4·5 mg., 40 μc), L-serine (24 mg.), dihydroPGA (9·5 mg.) and dialysed enzyme in a total volume of 3·5 ml. were incubated together under $N_2 + CO_2$ (95:5, v/v) in a Warburg flask at 37° for 20 min. The amount of enzyme used was sufficient to form 40 μmoles of serine in the standard system in this time. At the end of the incubation period the pteridines were separated as their barium salts by the procedure used in method (*b*) above. After removal of Ba^{2+} ions the solution was freeze-dried and a portion of the resultant yellow powder counted on lens tissue on a 1 cm.^{2} polythene disk under a conventional end-window Geiger–Müller tube. The result corresponded to 76300 counts/min./cm.^{2} at infinite thickness, under conditions in which the L-serine present in the medium would have given 450000 counts/min./cm.^{2} at infinite thickness.

A portion of the material was dissolved and chromatographed by the ascending technique on Whatman no. 4 paper with 0·1 M-K_2HPO_4 as solvent. Over ultraviolet light three blue-fluorescing areas were visible with R_F values of 0·17, 0·39 and 0·57, and in addition there was a yellow-fluorescing area of R_F 0·05. A radioautograph revealed no radioactivity in the two slowest-moving fluorescent spots but showed areas of weak radioactivity corresponding to the fluorescing areas of R_F 0·39 and 0·57. In addition, strong radioactivity was indicated at a non-fluorescent area of R_F 0·93, which was shown by control chromatograms to correspond to the position of serine. Under an end-window counter the areas of R_F 0·39, 0·57 and 0·93 had radioactivity of 15, 29 and 973 counts/min. above background, so that almost all the radioactivity was present in the fast-moving compound. This was probably a trace of serine which had been co-precipitated with the barium salt; it did not appear to be a pteridine since it was non-fluorescent and did not absorb ultraviolet light.

The significance of the very small amount of radioactivity associated with the fluorescent areas is doubtful, but the radioactivity was far below that expected for an intermediate in equilibrium with the serine β-carbon.

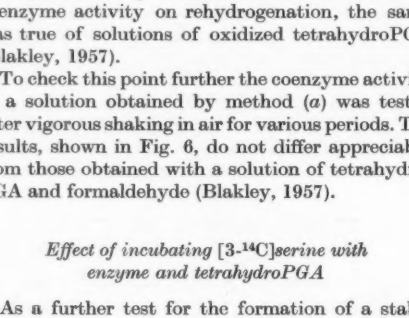

Fig. 6. Effect of aeration on the coenzyme activity of tetrahydroPGA after incubating with enzyme and formaldehyde. The reaction mixture, containing 3·33 mM tetrahydroPGA, 10^{-2} M formaldehyde and enzyme, was incubated under N_2 at 37° and then boiled. It was transferred to a test tube and shaken vigorously under air. At intervals, 0·3 ml. samples were removed and tested with the standard enzyme system. For further details, see text.

DISCUSSION

The enzyme system responsible for serine synthesis showed a normal Michaelis–Menten relationship between glycine concentration and reaction velocity except that the Michaelis constant K_m was found to have the rather high value of $2·8 \times 10^{-2}$ M. However, glycine forms a Schiff's base with pyridoxal phosphate before, or perhaps in the process of, combining

with the enzyme (Blakley, 1955), so that this result was perhaps not surprising. The reaction velocity did not increase uniformly with formaldehyde concentration, but diminished with increase of the formaldehyde concentration above 0·01 M (Table 1). Since formaldehyde may inactivate enzymes by combining with them and since tetrahydroPGA is also inactivated by excessive amounts of formaldehyde (Blakley, 1957), this result also conformed to expectation.

Although some liver preparations will convert sarcosine into serine, especially in the presence of added glycine (Mitoma & Greenberg, 1952), no synthesis of serine occurred when sarcosine was substituted for glycine in the enzyme system studied in the present work. It is possible that an entirely different enzyme system is responsible for the conversion of sarcosine into serine, but it seems likely that in crude preparations sarcosine is first converted into glycine and formaldehyde which then react in the system described in this paper.

Results on the specificity of the enzyme for tetrahydroPGA indicate that the 2-amino and 4-hydroxy substituents on the pteridine ring are essential for coenzyme activity. Although slight activity was obtained with 4-amino-4-deoxyPGA this was almost certainly due to contaminating PGA, an impurity difficult to remove. The glutamic acid residue of PGA is essential for activity and the results also suggest that reduced 'conjugates' of PGA possessing three or more glutamic residues are even more active than is tetrahydroPGA itself. Thus with tetrahydropteroyltriglutamate, as with tetrahydroPGA, the results conformed to the simple Michaelis–Menten relation and the respective values of K_m were $8{\cdot}3 \times 10^{-5}$ M and $1{\cdot}8 \times 10^{-4}$ M. Only a small quantity of tetrahydropteroylheptaglutamate was available for testing, but this also had good activity. It seems possible that the extended peptide chain of glutamic acid residues increases the rate of reaction of the coenzyme with the enzyme to form an active complex.

Experiments designed to demonstrate the existence of a pteridine intermediate in which tetrahydroPGA is combined with a one-carbon group which can be converted enzymically into the β-carbon of serine or into formaldehyde have been rather inconclusive. Paper chromatography revealed no new fluorescent or ultraviolet-light-absorbing compound, but the many compounds formed by oxidation of tetrahydroPGA may have obscured such a substance on the chromatogram. Solutions of the products from the incubation of enzyme and tetrahydroPGA with either formaldehyde or serine lose their coenzyme activity during aeration almost as rapidly as a simple solution of tetrahydroPGA and formaldehyde. Only slight amounts of radioactivity were incorporated into fluorescent pteridines when [3-^{14}C]serine was incubated with enzyme and dihydroPGA.

On the whole, therefore, the evidence favours the view that during serine synthesis by the enzyme system tetrahydroPGA forms only readily dissociating compounds, unstable to oxidation, such as hydroxymethyl derivatives.

SUMMARY

1. The rate of synthesis of serine from glycine and formaldehyde by a partially purified enzyme from rabbit liver was found to be proportional to enzyme concentration and time of incubation.

2. The reaction ultimately reached an equilibrium corresponding to a value of the constant [glycine] [formaldehyde]/[serine] of approximately 5·8 mM.

3. The relation between glycine concentration and reaction velocity conformed to the requirements of the simple Michaelis–Menten equation, giving a K_m of $2{\cdot}8 \times 10^{-2}$ M. The optimum formaldehyde concentration was 0·01 M, the reaction velocity diminishing at higher concentrations.

4. Hydrogenated pteroylglutamic acid (PGA), N^{10}-formylPGA, pteroyltriglutamate and pteroylheptaglutamate were the only pteridines active as intermediates among those tested. The 2-amino and 4-hydroxy groups, the hydrogenated pyrazine ring and one or more glutamic acid residues are essential for activity. Leucovorin, citrovorum factor and anhydroleucovorin A are all inactive.

5. Dihydro-2-deaminoPGA, but not tetrahydro-4-amino-4-deoxyPGA, is an inhibitor of serine synthesis with tetrahydroPGA as coenzyme.

6. The relations between reaction velocity and the concentration of dihydroPGA, tetrahydroPGA and tetrahydropteroyltriglutamate respectively conform to the requirements for simple Michaelis–Menten equations giving K_m values of $2{\cdot}9 \times 10^{-4}$ M, $1{\cdot}8 \times 10^{-4}$ M and $8{\cdot}3 \times 10^{-5}$ M respectively.

7. The reaction products from incubation of enzyme and tetrahydroPGA with serine or formaldehyde do not contain any new pteridine detectable by chromatography, and are inactivated by aeration at about the same rate as a simple mixture of solutions of tetrahydroPGA and formaldehyde.

8. Incubation of enzyme, dihydroPGA and [3-^{14}C]serine results in only very slight incorporation of radiocarbon into pteridines.

9. The nature of the pteridine intermediate of enzymic serine synthesis is discussed in terms of these results.

It is a pleasure to express my indebtedness to those mentioned above who supplied essential compounds for this work. In addition I wish to thank Professor A. H. Ennor for helpful advice and criticism.

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Biosynthesis of Fatty Acids in Cell-free Preparations

4. SYNTHESIS OF FATTY ACIDS FROM ACETATE BY A PARTIALLY PURIFIED ENZYME SYSTEM FROM RABBIT MAMMARY GLAND*

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It was shown in previous papers of this series (Popják & Tietz, 1955; Tietz & Popják, 1955) that cell-free extracts prepared from lactating-rat mammary gland were able to synthesize long- and short-chain fatty acids from [^{14}C]acetate. This synthesis was dependent on the addition of adenosine triphosphate (ATP), the concomitant oxidation of pyruvate, oxaloacetate or α-oxoglutarate, and the presence of coenzyme A (CoA). This last requirement could be demonstrated only if the extracts were depleted of endogenous CoA by treatment with Dowex-I resin.

Before commencing a detailed study of the steps involved in fatty acid synthesis, it was necessary to simplify the original assay conditions used by Popják & Tietz (1955), and to attempt the purification of the mammary gland extracts. In this communication the preparation of a partially purified enzyme system from rabbit mammary gland is described. This system can synthesize fatty acids from acetate in the presence of stoicheiometric amounts of reduced diphosphopyridine nucleotide (DPNH) as electron donor. The results have been presented in a preliminary form (Hele & Popják, 1955).

MATERIALS AND METHODS

Preparation of the fatty acid-synthesizing fraction from rabbit mammary gland

Lactating rabbits, about 17 days after parturition, were killed by the intravenous injection of Nembutal (10%, w/v; 1·5 ml./kg.) and the mammary glands excised. The average yield was 100 g./animal. The glands were cooled in crushed ice, freed from fat and connective tissue, and finely minced with scissors. The mince was homogenized for 2 min. in a blender (M.S.E. Ato-mix 800), run at top speed, with 2 vol. of buffer (0·154 M-KCl, 100 parts; 0·154 M-$MgCl_2$, 10 parts; 0·1 M potassium phosphate buffer, pH 7·4, 34 parts). This and all subsequent operations were carried out at 0–4°. The homogenate was centrifuged at 400 *g* for 10 min., and the supernatant collected with filtration through a pad of gauze to remove fat. This supernatant was centrifuged at 44000 *g* for 1 hr. in the Spinco model L preparative ultracentrifuge. In the earliest preparations the supernatant was centrifuged once again, this time at 100000 *g* for 1 hr., before precipitation with $(NH_4)_2SO_4$ was carried out. In later preparations, when much larger volumes of material were being handled, this second centrifuging was dispensed with and precipitation with $(NH_4)_2SO_4$ carried out in the following manner with the material centrifuged at 44000 *g*.

The supernatant was brought to 90% saturation by the addition of 70 g. of $(NH_4)_2SO_4$/100 ml. at 0–4°. The precipitate (F 90) was collected by filtration on a large fluted paper (Whatman no. 12) overnight, followed by press-drying between filter papers the next day. This precipitate could be stored in solid CO_2 for at least 6 months with no loss of fatty acid-synthesizing activity.

Refractionation of this material was carried out as follows. Sufficient cold water was added to each 21 g. of F 90 precipitate to bring the volume to 100 ml., and saturated with $(NH_4)_2SO_4$ to about 21%. A smooth suspension was achieved by mechanical stirring. Ammonium sulphate (9·8 g./100 ml.) was added to give a final saturation of about 35%; the precipitate was removed by centrifuging at 100000 *g* for 10 min. in the Spinco ultracentrifuge. This high-speed centrifuging was necessary for the removal of finely particulate material which could not be removed in 20 min. at 40000 *g*.

A clear supernatant was obtained, which was brought to about 60% saturation by the addition of 17·4 g. of $(NH_4)_2SO_4$/100 ml. of supernatant, and the precipitate collected by centrifuging at 22000 *g* for 10 min. This precipitate, which

* Part 3: Tietz & Popják (1955).

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contained the fatty acid-synthesizing system, was dissolved in 0·05 M-$KHCO_3$ to give a final protein concentration of 50–70 mg./ml. and will be referred to as the fatty acid-synthesizing fraction (FASF). About 1 g. of protein was obtained in this fraction from 100 g. of mammary-gland tissue. These preparations, if undialysed, retained their fatty acid-synthesizing activity for 2–3 months if kept frozen at −15°.

For certain experiments the FASF was dialysed for 15–18 hr. at 0–4° against 0·05 M aminotrishydroxymethylmethane (tris) buffer, pH 7·5, containing 10^{-3} M cysteine; 1 l. of buffer was used/ml. of FASF. Some precipitation of protein occurred, and the dialysed preparations were centrifuged at 20 000 *g* for 10 min. immediately after dialysis to remove this material. Such dialysed preparations lost 10–30% of their activity immediately after dialysis. They became totally inactive after 1–2 weeks, even when stored at −15°, at a protein concentration of about 50 mg./ml. and in the presence of small amounts of $(NH_4)_2SO_4$ added immediately after dialysis.

The fractions obtained from different batches of mammary gland were far more constant in their fatty acid-synthesizing ability than were the rat mammary-gland extracts previously employed.

Assay systems

The following basic system was used with little modification both for experiments in which DPNH utilization was studied in the spectrophotometer (Unicam SP. 500), and for work upon the incorporation of ^{14}C-labelled acetate into fatty acids and fatty acylhydroxamates. Each 3 ml. of reaction mixture contained: 30 μmoles of ATP, 2 μmoles of CoA, 0·4–0·8 μmole of DPNH, 30 μmoles of cysteine, 100 μmoles of tris buffer, pH 9·4; 20 μmoles of $MgCl_2$; 5–60 μmoles of acetate (labelled or unlabelled) and 5–10 mg. of FASF. The final pH was 8. All substances were neutralized with KOH or HCl as indicated. All the reagents were incubated together without acetate, or without FASF, at 38° for 5 min. and the reaction was started by the addition of the missing component. All experiments were performed in test tubes, small conical flasks, Warburg flasks, or spectrophotometer cells, with no precautions taken to obtain anaerobic conditions. Most experiments done in the spectrophotometer were carried out at room temperature after the initial incubation, but a few experiments were performed at 38° with an electrically heated, thermostatically controlled cell-carrier to be described elsewhere. Additions to spectrophotometer cells were made with the adder-mixer described by Boyer & Segal (1954).

Extraction of fatty acids

This was done as previously described (Popják & Tietz, 1954; 1955).

Chromatographic fractionation of the fatty acids from C_3 to C_{18} was done by the reversed-phase technique of Howard & Martin (1950) and, when separation of butyric, crotonic (*trans*-but-2-enoic), hexanoic and octanoic acids was required, by the liquid–vapour chromatography of James & Martin (1952).

[*carboxy*-^{14}C]Acetate was obtained from the Radiochemical Centre, Amersham, Bucks., and was usually diluted with unlabelled potassium acetate to a specific activity of 1 μc/μmole. The methods of purifying the fatty acids from radioactive acetate, the methods of ^{14}C assay and calculations employed, have already been described (Popják & Tietz, 1954, 1955).

Preparation and chromatography of hydroxamates from acyl-CoA derivatives

The method of Lipmann & Tuttle (1945) was used, except that salt-free hydroxylamine prepared by the method of Beinert *et al.* (1953) was employed. A solution (1 vol.) of 2 M hydroxylamine in methanol (pH 6·5) was mixed with 1 vol. of 0·1 M acetate buffer (pH 5·4), and added to 1 vol. (usually 3 ml.) of the enzymic incubation mixture. After 1 hr. at about 20°, the mixture was evaporated to dryness under reduced pressure on a hot-water bath; the hydroxamates were extracted from the dry residue with warm ethanol (5 ml.). The ethanolic extract was evaporated to dryness under reduced pressure in a small tube and the hydroxamates were taken up in 0·2–0·3 ml. of ethanol; insoluble salts were removed by centrifuging at 0°. Samples (0·01–0·05 ml.) were placed in small spots on strips of Whatman no. 1 paper and chromatographed according to Thompson (1951) with either butanol–acetic acid–water (4:1:5) or octanol–formic acid–water (3:1:3) solvent systems. Marker hydroxamates were prepared from methyl or ethyl esters of the various acids or from the acid anhydrides. With the two solvent systems it has been possible to achieve satisfactory resolution (in order of increasing R_F values) of the hydroxamates of acetoacetic, β-hydroxybutyric, acetic, (?β-oxohexanoic), crotonic, butyric, hexanoic, β-hydroxyoctanoic and octanoic acids. Only the acetohydroxamate and β-hydroxybutyrohydroxamate were present in sufficient quantity in the samples applied to the paper to give intensely coloured spots on the paper after spraying with 5% $FeCl_3$ soln. in 0·1 N-HCl. Hydroxamate prepared from ethyl acetoacetate gives no colour with the acid $FeCl_3$ soln.; it reacts, however, with neutral $FeCl_3$ but the intensity of colour on a molar basis is only about one-fiftieth of that given by acetohydroxamic acid.

The position and amounts of [^{14}C]hydroxamates on the paper strips were determined by an automatic recording scanning device in which the chromatographic paper strips were driven between two thin mica-window Geiger–Müller counters. The pulses from the counters were taken to a ratemeter and were recorded simultaneously at four different levels of sensitivity varying from 3^0 to 3^3; i.e. if the counting rate at the lowest sensitivity of the instrument gave a galvanometer deflexion of 1 cm., the same counting rate was recorded simultaneously also as 3, 9 and 27 cm. deflexions. The chart of the recording galvanometer ran synchronously with the movement of the chromatogram between the Geiger counters. Thus the position of each spot on the paper, even in the absence of a colour reaction, could easily be determined. For identification purposes hydroxamates prepared from the enzyme incubations were mixed with authentic samples of hydroxamates and chromatographed; the chromatograms were sprayed with a 5% $FeCl_3$ soln. in 0·1 N-HCl and were then scanned for radioactive spots. It was found experimentally that the areas under the curves obtained in these records were proportional to the concentration of ^{14}C-labelled material in the spots. From the measurement of the areas under the curves the total radioactivity of each spot could be determined with an accuracy of about 20%. For more accurate evaluations

appropriate portions of the chromatograms were cut into equal squares and their ^{14}C content was measured individually by counting under a mica-window Geiger counter.

Materials

CoA (about 75% pure, batches 405, 407, 408) and diphosphopyridine nucleotide (DPN, 92% β-isomer) were obtained from the Pabst Laboratories. DPNH (about 64% of reduced coenzyme) and highly purified yeast alcohol-dehydrogenase were obtained from Boehringer & Soehne. ATP was obtained from L. Light and Co. or from the Sigma Chemical Co. (98–99% pure). Triphosphopyridine nucleotide (TPN, 98% pure), adenosine 5-phosphate (AMP, 98–100% pure), glucose 6-phosphate (98–100% pure), TPN-specific glucose 6-phosphate dehydrogenase and hypoxanthine were also Sigma preparations. Tris buffer (L. Light and Co.) was purified by recrystallization from aqueous ethanol. Diol buffer (2-amino-2-methylpropane-1:3-diol; L. Light and Co.) was not recrystallized.

Reduced triphosphopyridine nucleotide (TPNH) was prepared from Sigma TPN, by the method of Conn, Kraemer, Liu & Vennesland (1952). Assays of this preparation showed, per mg.: 0·48 μmole of total TPN nucleotide (about 0·36 mg.) by adenine determination at 260 mμ.; 0·23 μmole of TPNH, from the optical density at 340 mμ. and confirmed by TPNH–glutathione reductase assay; 0·28 μmole of TPN, determined by glucose 6-phosphate dehydrogenase assay. The nucleotide was therefore 48% reduced. No DPN or DPNH could be detected by alcohol dehydrogenase assay.

Oxidized glutathione (GSSG) was prepared by the oxidation with iodine of the partially reduced substance (Hopkin and Williams) followed by precipitation with an acetone–ether mixture and drying *in vacuo*. TPNH–glutathione reductase was prepared from peas, according to Mapson & Goddard (1951), and refractionated with $(NH_4)_2SO_4$ (Kaplan, Colowick & Neufeld, 1953). Dowex resins, nos. 1 and 50, came from the Dow Chemical Co., Midland, Michigan, U.S.A.

Chemical methods

Protein was determined by the biuret procedure of Gornall, Bardawill & David (1949) which agreed to within 10% with the dry weight method used by Popják & Tietz (1954). Hydroxamates were determined according to Lipmann & Tuttle (1945) and orthophosphate according to Allen (1940).

Pyridine nucleotides. In studies of interconversions and destructions of the reduced and oxidized forms of these nucleotides TPN was determined by the use of glucose 6-phosphate dehydrogenase and TPNH by the TPN-specific glutathione reductase (Mapson & Goddard, 1951) present in the FASF. DPN and DPNH were assayed with alcohol dehydrogenase (Racker, 1950) with ethanol or acetaldehyde respectively as the substrate. In the latter case the pH of the incubation mixture (which under conditions of fatty acid synthesis was pH 8·0) was lowered by carefully controlled addition of M phosphate buffer, pH 6·5, in order to shift the equilibrium of the alcohol dehydrogenase reaction further towards the oxidation of DPNH. In all these instances the change observed in the optical density of the solutions at 340 mμ. was used as a quantitative measure of the assays. The molar extinction coefficient of DPNH (and TPNH) was taken to be $6{\cdot}22 \times 10^6$ cm.^{2} × mole^{-1} (Horecker & Kornberg, 1948).

RESULTS

Incorporation of [^{14}C]acetate into fatty acids by preparations of rabbit mammary gland

The rabbit mammary-gland extract, when tested in a system similar to that used by Popják & Tietz (1954) and before treatment with ammonium sulphate, showed a fatty acid-synthesizing activity (Table 1) close to that obtained with rat mammary-gland extracts. The same requirements for cofactors and other additions were shown. α-Oxoglutarate and malonate did not stimulate synthesis by the rabbit preparation to the extent obtained with rat mammary-gland extracts. Since it was thought that the stimulating action of α-oxoglutarate in the presence of DPN on fatty acid synthesis was due to the generation of DPNH (Tietz & Popják, 1955) we simplified the assay system by using DPNH alone. As is shown in Table 2 the use of DPNH with a dialysed and undialysed enzyme preparation was nearly as effective as DPN + α-oxoglutarate + malonate in promoting fatty acid synthesis. An almost complete dependence of fatty acid synthesis on

Table 1. *Fatty acid synthesis from [^{14}C]acetate by mammary-gland extract of rabbits*

Each flask contained: potassium acetate, 60 μmoles (5 μc of ^{14}C); CoA, 0·3 μmole; DPN, 1 mg.; undialysed mammary-gland extract, 2 ml. Final vol., 3 ml. Incubation for 1 hr. at 38°.

Additions	Acetate used for fatty acid synthesis/100 mg. of protein (μm-moles)
None	7·37
ATP, 0·01 M	173·00
α-Oxoglutarate, 0·01 M	25·40
Malonate, 0·05 M	42·20
α-Oxoglutarate + malonate	23·80
α-Oxoglutarate + ATP	126·00
Malonate + ATP	234·00
α-Oxoglutarate + malonate + ATP	286·00

Table 2. *Fatty acid synthesis in dialysed and undialysed rabbit mammary-gland extracts*

Each flask contained: ATP, 30 μmoles; potassium acetate, 60 μmoles (5 μc of ^{14}C); CoA, 0·3 μmole. Final vol., 3 ml. Incubation for 1 hr. at 38°. Fatty acid synthesis expressed as μmole of acetate incorporated into fatty acids/100 mg. of protein.

Additions	Fatty acid synthesis: Undialysed enzyme	Fatty acid synthesis: Dialysed enzyme
DPN (1 mg.)	0·270	0·072
DPN + α-oxoglutarate (30 μmoles) + malonate (150 μmoles)	0·438	0·256
DPNH* (1 mg.)	0·357	0·192

* Prepared by chemical reduction of DPN.

DPNH was demonstrated after refractionation of the mammary-gland extracts with ammonium sulphate.

As is shown in Table 3 the refractionation of F 90 to FASF increased the synthesizing activity some eight- to ten-fold, and at the same time removed endogenous DPN, so that a marked requirement for this coenzyme could be demonstrated. In all FASF preparations there was a small, yet significant, utilization of acetate for fatty acid synthesis (about 0·005 μmole/10 mg. of protein/60 min.) even in the absence of added DPNH. This blank could not be reduced further either by dialysis or by treatment of dialysed preparations with Dowex-1 resin (cf. Table 4). This suggested that small amounts of DPN or DPNH might be added with the CoA or ATP, but assay with alcohol dehydrogenase failed to reveal such contamination.

Langdon (1955) suggested that the optimum incorporation of [^{14}C]acetate into fatty acids by rat-liver extracts is dependent upon TPNH rather than upon DPNH. The synthesis achieved in Langdon's experiments in the presence of TPNH (0·6 μmole/3 ml. of reaction mixture) was double that obtained in its absence. This raised the possibility that the FASF might also show a requirement for TPN or TPNH and that the fatty acid synthesis observed by us on addition of DPNH might be accounted for by the presence of a transhydrogenase (Kaplan *et al.* 1953) which would reduce endogenous TPN with DPNH. These possibilities were tested in the experiments presented in Table 4. In one set of experiments chemically prepared TPNH was used, and in another TPNH was generated from TPN by the action of the TPN-specific glucose 6-phosphate dehydrogenase present (in non-rate-limiting amounts) in the FASF. These experiments demonstrated that the addition of stoicheiometric amounts of TPNH had no stimulatory effect upon fatty acid synthesis by the rabbit preparation, and provided no evidence for the presence of transhydrogenase in the FASF.

The breakdown of TPN or TPNH either through dephosphorylation, or by other pathways, was small under the experimental conditions (i.e. in the presence of all the cofactors required for fatty acid synthesis). It was insufficient to account for the negative effect of TPNH on fatty acid synthesis on the basis of the destruction of TPN or TPNH; but a small conversion into DPN or DPNH was observed in control experiments, sufficient to account for the [C^{14}]acetate incorporations in the absence of DPNH. DPN could be reduced to DPNH by

Table 3. *Fatty acid synthesis from* [^{14}C]*acetate by ammonium sulphate fractions of mammary-gland extract in the presence and absence of DPNH*

The incubation mixture, in a final vol. of 3 ml., contained: ATP, 30 μmoles; CoA, 1·5 μmoles; cysteine, 30 μmoles; potassium acetate, 60 μmoles (= 11 μc of ^{14}C); $MgCl_2$, 20 μmoles; tris buffer, pH 9·4, 100 μmoles. In the experiment with F 90, 18·5 mg. of protein, and in the other two (FASF) 10·8 mg. of protein, were used. DPNH (0·5 μmole) was added. Incubation at 38°. Fatty acid synthesis is expressed in terms of acetate μm-moles utilized/10 mg. of protein.

Ammonium sulphate fraction	Length of incubation (min.)	Fatty acid synthesis: DPNH added	Fatty acid synthesis: No DPNH
F 90	27	3·56	1·90
FASF*	10·5	10·60	0·49
FASF*	21	23·05	3·04

* FASF was made from the F 90 shown in this table.

Table 4. *Fatty acid synthesis from* [^{14}C]*acetate. Tests for specificity of pyridine nucleotides*

The standard assay system described under Methods was used with 10 μmoles (10 μc of ^{14}C) of potassium acetate and 0·8 μmole of the pyridine nucleotides. The reaction mixtures were incubated with 5 or 10 mg. of protein at 38° for 30 min. (Expts. 1 and 2) and 45 min. (Expt. 3). The FASF preparations were obtained at different times from the same F 90.

Pyridine nucleotide added	Acetate (μmoles) incorporated into fatty acids: Expt. 1, Undialysed FASF (10 mg.)	Expt. 1, Dialysed FASF (10 mg.)	Expt. 2* Dialysed FASF (10 mg.)	Expt. 3* Dialysed FASF (5 mg.)
None	0·006	0·005	0·006 (0·010)†	—
DPNH	0·342	0·233	0·162 (0·132)†	0·344
DPNH + TPN	0·334	0·248	0·200	—
TPNH	—	—	0·023	0·029
DPNH + TPNH	—	—	0·150	0·310
DPN + TPNH	—	—	0·024	0·018
TPN	—	—	—	0·007
DPN	—	—	—	0·030

* In Expt. 2 the TPNH was prepared by chemical reduction; in Expt. 3 the TPNH was generated in the FASF by the addition of glucose 6-phosphate (1 μmole) at the start of incubation.

† The figures in parentheses relate to results obtained with dialysed and Dowex-treated enzyme.

AMP, itself generated from ATP during the activation of acetate (Lipmann, Jones, Black & Flynn, 1952; Beinert *et al.* 1953). The relevant data are presented below.

Utilization of DPNH associated with fatty acid synthesis

The FASF contained a small amount of reducible substrate, which caused a blank utilization of DPNH in the absence of acetate. Because this substrate (or substrates) could not entirely be removed by dialysis and because the FASF was partially inactivated by dialysis, it was finally decided to exhaust the reducible material, at the expense of the loss of a certain amount of DPNH, by pre-incubation of the reaction mixture at 38° for 5 min. in the absence of acetate. The loss of DPNH during such period, as measured by the fall in optical density at 340 mμ., varied in different preparations from 5 to 20% of the amount of DPNH added. After incubation the optical density of the reaction mixture was read in the spectrophotometer until the fall ceased; this usually occurred within 5 min. When the optical density readings were steady, acetate was added, whereupon a rapid, progressive drop in optical density occurred. A drop of this magnitude was not observed in the control, to which water had been added (Fig. 1). For the first 5 min. the rate of DPNH utilization was linear with time; then the reaction slowed down, reaching a steady state when about 60% of the DPNH had been utilized. The reaction was resumed when more DPNH was added, and, as before, reached a steady state at 60% DPNH utilization. The addition of more CoA, acetate, or ATP did not lead to a similar resumption of the reaction. Addition of more FASF was either without effect, or resulted in a slow, indecisive drift forwards or backwards.

In Fig. 1 it will be noted that upon the addition of acetate or water a small rise in optical density (about 0·04 unit) occurred. This was due to the disturbance of fine particles added with the FASF, which were not completely removed by centrifuging and were believed to be fatty in nature. This rise was observed in many experiments, but in as many others a fall in optical density occurred. Since it was difficult to ascertain the exact time at which DPNH utilization commenced, the reading observed 30 sec. after making the additions was taken as that for zero time, and DPNH utilization was measured in terms of decrease in optical density from this point. A small error was inevitably introduced, but this could be rendered proportionately smaller by using larger amounts of DPNH (about 0·8 μmole), thereby extending the linear part of the curve for up to 20 min. The percentage utilization of DPNH was calculated on the basis of the amount of DPNH present at zero time, obtained by subtracting a similarly treated blank (which contained all reagents except DPNH) from the zero-time reading.

DPNH utilization in the presence of acetate could also be demonstrated in the F 90 precipitate (Table 5). Refractionation with ammonium sulphate led to a fourfold increase in acetate-de-

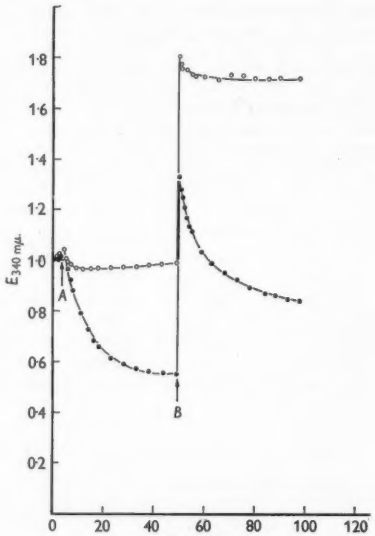

Fig. 1. Utilization of DPNH by the FASF. Each cell contained the standard assay system with 0·4 μmole of DPNH. At *A* 50 μmoles of acetate was added to ●, and an equivalent volume of water to ○. At *B* a further 0·4 μmole of DPNH was added to both cells.

Table 5. *Purification of the fatty acid-synthesizing system based on acetate-dependent utilization of DPNH*

The assay system, in a final vol. of 3 ml., contained: ATP, 30 μmoles; CoA, 1·5 μmoles; DPNH, 0·6 μmole; cysteine, 30 μmoles; tris buffer, pH 9·4, 100 μmoles; potassium acetate, 50 μmoles; $MgCl_2$, 20 μmoles; F 90, 19 mg. or FASF, 10·8 mg. (both undialysed). Assays at room temperature. 1 unit = 1 μmole of DPNH utilized in the presence of acetate/15 min.

Ammonium sulphate fraction	Total protein (mg.)	Total units	Units/10 mg. of protein	Recovery (%)	
				Protein	Units
F 90	860	2·56	0·03	100	100
FASF	190	2·42	0·13	22	95

pendent DPNH utilization per mg. of protein, with a 95 % recovery of this activity in the FASF.

The utilization of DPNH by the FASF was dependent on the presence not only of acetate, but also of CoA and ATP (Table 6). In the absence of cysteine only 30 % activity was observed. Little stimulation by magnesium was observed with the undialysed preparation used here.

The rate of DPNH utilization, when measured over the linear part of the curve, was dependent on the concentration of acetate, protein, CoA and ATP (Fig. 2). At low concentrations of protein or of CoA, lag phases were observed at the beginning of the reaction but the rates then increased, and linear portions were always found. Variation in protein concentration was without effect upon the point at which the steady state was reached; but this point varied with the concentrations of CoA and ATP in a complex manner that was not fully investigated.

The steady state was always reached when the same percentage of DPNH had been utilized, independent of the amount of DPNH present at zero time. Typical curves obtained with 0·290 and 0·656 μmole of DPNH (amounts present at zero time) are shown in Fig. 3. The steady state was reached when 0·179 and 0·395 μmole of DPNH had disappeared, giving 61·6 and 60·2 % utilization respectively. The rate of the reaction within the first 5 min. was independent of DPNH concentration.

It was thought possible that the steady state might represent the setting up of a true equilibrium of the reaction catalysed by the β-hydroxybutyryl dehydrogenase. (For terminology of enzymes of fatty acid metabolism see Beinert *et al.* 1956). This equilibrium is known to have a marked dependence upon pH (Lynen, Wessely, Wieland & Rueff, 1952; Wakil, Green, Mii & Mahler, 1954), and therefore the effect of pH upon the point at which the steady state

Table 6. *Component requirements of the spectrophotometric-assay system*

The standard assay system was employed with omissions as indicated. Undialysed FASF (10 mg.) was used and observations were made at room temperature.

	DPNH used/10 min./ 10 mg. of protein (μmole)
Complete system	0·147
No $MgCl_2$	0·127
No acetate	0·002
No CoA	0·004
No ATP	0·017
No cysteine	0·058
No enzyme	0·020

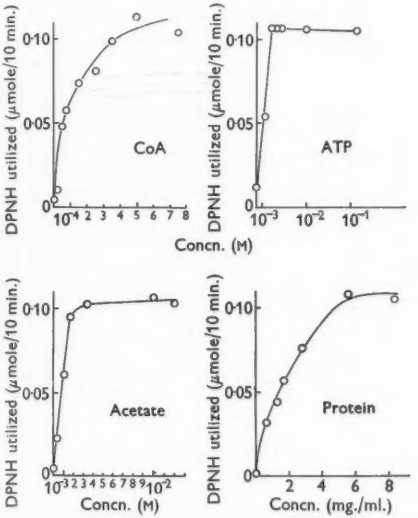

Fig. 2. Effect of the concentration of CoA, ATP, acetate and FASF upon the rate of DPNH utilization by the FASF. The standard assay system was used, with 0·8 μmole of DPNH.

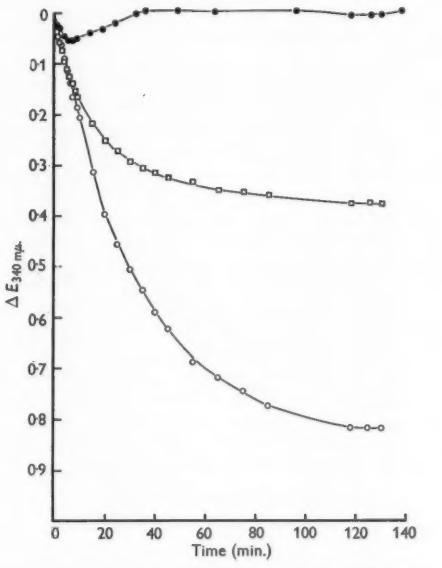

Fig. 3. Effect of DPNH concentration upon DPNH utilization by the FASF. The standard assay system was used, with 0·4 and 0·8 μmole of DPNH added initially. These amounts fell to 0·290 μmole (□) and 0·656 μmole (○) after the preliminary incubation at 38°. ●, 0·275 μmole of DPNH, no acetate, water added at zero time.

was established was investigated (Fig. 4). The experiments were run until no further drop in optical density occurred for at least 20 min. In a number of experiments slow re-reduction of DPN occurred after this time, possibly at the expense of AMP. The point at which the steady state occurred was expressed in terms of the percentage of DPNH unchanged (based on the DPNH present at zero time, as described above) when the reaction came to a standstill. This was done so that a direct comparison with the data of Wakil *et al.* (1954) could be obtained. The relevant equilibrium data of these workers is expressed in terms of percentage substrate (β-hydroxybutyryl-CoA) oxidized. Control experiments showed that tris and diol buffers were without effect upon DPNH utilization, other than the effect due to pH. All experiments were accompanied by a blank to which water was added in place of acetate. The pH of the reaction mixture was determined with the glass electrode at the end of the experiment.

The point at which the steady state was reached varied over the range pH 8–9·8. At pH 8, the percentage of DPNH remaining unchanged when the reaction came to a standstill was less than one-half of the percentage of DPNH unchanged at pH 9·3. Below pH 8, the blank oxidation of DPNH interfered with the interpretation of the results, but as the significance of this blank is difficult to assess, curves both corrected and uncorrected for it are shown.

These experiments show that between pH 8 and 9·8 a correlation exists between the effect of pH upon the steady state in the FASF system and upon the equilibrium of the reaction catalysed by the β-hydroxybutyryl dehydrogenase. It is concluded that the point at which the steady state is established is determined in the first place by the equilibrium of the reaction catalysed by β-hydroxybutyryl dehydrogenase present in the FASF. Other slower reactions, either utilizing or regenerating DPNH, are probably superimposed upon this, when the rate of DPNH utilization in the fatty acid-synthesizing system is sufficiently slowed down by the approach of the true equilibrium of the β-hydroxybutyryl dehydrogenase reaction.

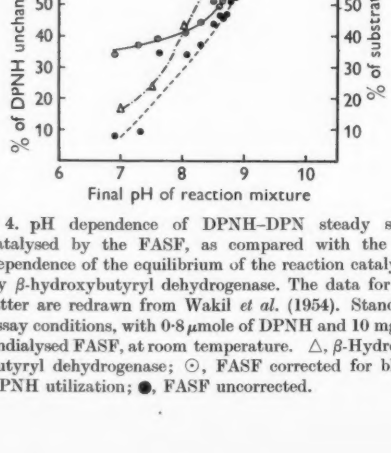

Fig. 4. pH dependence of DPNH–DPN steady state catalysed by the FASF, as compared with the pH dependence of the equilibrium of the reaction catalysed by β-hydroxybutyryl dehydrogenase. The data for the latter are redrawn from Wakil *et al.* (1954). Standard assay conditions, with 0·8 μmole of DPNH and 10 mg. of undialysed FASF, at room temperature. △, β-Hydroxybutyryl dehydrogenase; ⊙, FASF corrected for blank DPNH utilization; ●, FASF uncorrected.

Relationship between DPNH utilization and [C^{14}]*-acetate incorporation into fatty acids and fatty acid precursors*

In a number of experiments an attempt was made to examine the stoicheiometric relationship between DPNH utilization and [^{14}C]acetate incorporation into saturated and unsaturated fatty acids. For this purpose reaction mixtures of 30–60 ml. were employed. A sample was removed before the addition of [^{14}C]acetate, and the optical density at 340 mμ. was observed throughout the experimental period. After the addition of acetate, 3 ml. samples were withdrawn at various intervals, read in the

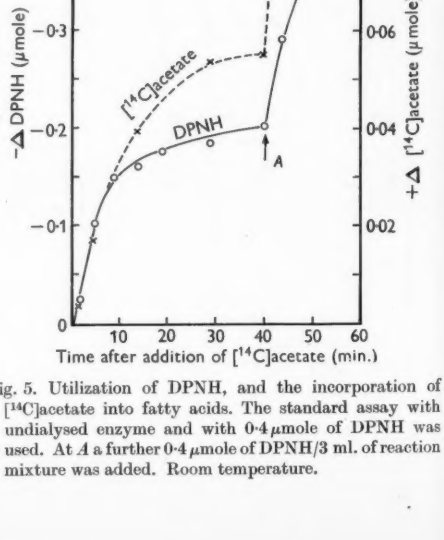

Fig. 5. Utilization of DPNH, and the incorporation of [^{14}C]acetate into fatty acids. The standard assay with undialysed enzyme and with 0·4 μmole of DPNH was used. At *A* a further 0·4 μmole of DPNH/3 ml. of reaction mixture was added. Room temperature.

spectrophotometer, and immediately saponified with KOH in preparation for the extraction and assay of labelled fatty acids. In some experiments further samples were taken for the estimation of hydroxamates by paper chromatography (see below).

The course of [^{14}C]acetate incorporation into saturated and unsaturated fatty acids followed closely the course of DPNH utilization (Fig. 5). During the early part of the experiment the ratio of DPNH utilized to [^{14}C]acetate incorporated was 5·7:1. This ratio fell to 3·3:1 when the steady state had been established. After the further addition of DPNH the ratio fell further to 2·4:1 and then rose again to 2·8:1.

Experiments of this type were of little use in obtaining data on the stoicheiometry of the reaction, although they suggested strongly that a precursor of fatty acids was being formed at the expense of DPNH and that this precursor was not extracted and estimated as fatty acid. The rate of formation of this precursor probably fell off as the steady state appeared, whereas the rate of fatty acid synthesis fell off more slowly. Further addition of DPNH, as is shown in Fig. 5, shifted the whole system in the direction of fatty acid synthesis.

Identification of fatty acids and their precursors by chromatography as their [^{14}C]*hydroxamates*

Two experiments were carried out, similar in principle to that shown in Fig. 5, except that the samples withdrawn at intervals were treated with the hydroxylamine reagent of Lipmann & Tuttle (1945), chromatographed, and scanned for radioactivity. The hydroxylamine reagent is expected to react with all fatty acids or their precursors present as their acyl–CoA derivatives.

In the first large-scale experiment, performed at room temperature, the chromatograms were developed with the octanol–formic acid solvent system. Samples were removed at the following intervals after the addition of acetate: at 5 min. during the linear phase of DPNH utilization; at 15 min. at the inflexion of the DPNH-utilization curve; at 45 min., some 15 min. after the steady state in DPNH utilization had appeared; at 55 min., i.e. 5 min. after the second addition of DPNH, and on the linear portion of the utilization curve; at 115 min., 65 min. after the second addition of DPNH and 15 min. after the setting up of the second steady state. DPNH utilization was observed throughout the experiment on a 3 ml. sample withdrawn from the main digest immediately after the addition of acetate, and was accompanied by a blank sample withdrawn before the addition of acetate. The data on the distribution of radioactivity amongst the fatty acyls* and their precursors were expressed as μmoles of these substances formed, and published in a preliminary communication (Hele & Popják, 1956). To facilitate the present discussion of this experiment figures from this preliminary communication will be included in the text where necessary.

The octanol–formic acid solvent system used in this experiment gave excellent separation of the hydroxamates of the short-chain fatty acids, but did not differentiate between β-hydroxybutyryl and acetoacetyl. The percentage of β-hydroxybutyryl present in this mixed component was later calculated to be about 30, on the basis of information obtained from chromatograms from a second experiment, which were developed with the butanol–acetic acid solvent system. This system gives a very satisfactory separation of the two acids, and a direct measure of the ratio of β-hydroxybutyryl to acetoacetyl (Table 8). Percentages arrived at in this manner are considered preferable to those given in the preliminary communication (Hele & Popják, 1956), which were obtained by calculation from the overall utilization of DPNH and incorporation of [^{14}C]acetate, and based on the arbitrary assumption that perfect stoicheiometric agreement between these two could be obtained experimentally.

The first experiment showed that acetyl, and β-hydroxybutyryl plus acetoacetyl, were present throughout in large excess over the higher fatty acyls and their precursors. Even if β-hydroxybutyryl represents only 30% of the mixed component it is still present in considerable excess over crotonyl. This may be illustrated by making a comparison between the figures for β-hydroxybutyryl and acetoacetyl in Table 7, and the figures for higher fatty acyls also taken from the preliminary communication (Hele & Popják, 1956). It is unlikely that the amounts of acetyl, β-hydroxybutyryl and acetoacetyl present are at any time low enough to be rate-limiting. This excess of β-hydroxybutyryl over fatty acyls, particularly during the early part of the experiment, but falling off as more fatty acyls are formed, probably accounts for the excess of DPNH utilization over [^{14}C]acetate incorporation observed in experiments of the type shown in Fig. 5, and strongly suggests that the postulated precursor in these experiments was β-hydroxybutyrate.

These chromatograms also showed clearly the stepwise synthesis of fatty acids, as originally postulated by Lynen (1954). After 5 min. crotonyl (0·005 μmole), butyryl (0·01 μmole), and a substance identified by its R_F as β-hydroxyoctanoyl (0·0025 μmole), appeared. No higher fatty acyls

* Since these acids are present as their hydroxamates, which must in turn have been derived from acyl–CoA derivatives, these acids are referred to as their acyl radicals.

were found. After a further 10 min. the amounts (μmoles) of crotonyl (0·017), butyryl (0·032) and β-hydroxyoctanoyl (0·0065) present had approximately trebled, and a trace of octanoyl (0·0015) had appeared. After 45 min. further increases in these substances had occurred, amounting to 0·026, 0·082, 0·03 and 0·0065 μmole respectively. Five minutes after the second addition of DPNH the amount of butyryl and octanoyl had increased to 0·111 and 0·013 μmole, the amount of crotonyl decreased to 0·018 μmole, and the amount of β-hydroxyoctanoyl increased to 0·013 μmole. These amounts were relatively unchanged by the time the second steady state had become established, but hexanoyl had appeared for the first time, suggesting that this system preferentially metabolized C_6 derivatives, thus preventing their accumulation.

Table 7. *Stoicheiometry of fatty acid synthesis from DPNH and* [^{14}C]*acetate*

The standard assay system was used with 10 mg. of undialysed FASF and 0·8 μmole of DPNH/3 ml. of incubation mixture; 50 min. after addition of acetate another 0·4 μmole of DPNH/3 ml. was added. (A): μmole of β-hydroxybutyryl + acetoacetyl formed (Hele & Popják, 1956). (B): μmole of β-hydroxybutyryl formed, obtained by correcting the data in column (A) (see text). These figures are taken as equivalent to the μmoles of DPNH utilized in the formation of β-hydroxybutyryl. (C): μmole of DPNH utilized in the formation of butyryl, crotonyl, β-hydroxyoctanoyl, and octanoyl, calculated from data given by Hele & Popják (1956). (D): DPNH utilized (μmole), as measured at 340 mμ., and corrected for blank utilization in a system without acetate. (E): DPNH calc. [columns (B) + (C)]/DPNH utilized, 340 mμ.

Time after addition of acetate (min.)	(A)	(B)	(C)	(D)	(E)
15	0·31	0·122	0·123	0·300	0·82
45	0·57	0·150	0·379	0·362	1·46
55	0·58	0·153	0·393	0·406	1·34
115	0·55	0·152	0·328	0·698	0·69

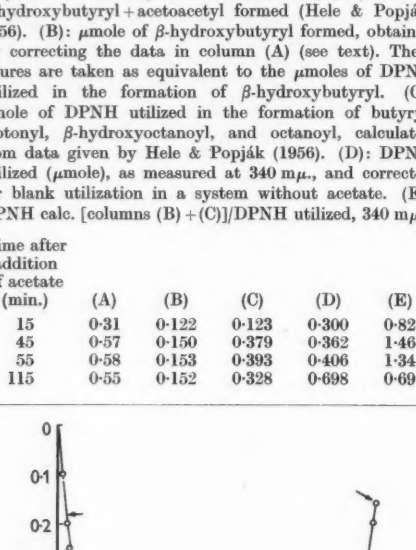

Fig. 6. Progress of DPNH utilization during an extended period of fatty acid synthesis. Experiment from which results shown in Table 8 have been derived. Standard conditions of assay with 10 mg. of undialysed FASF, 0·4 μmole of DPNH and 10 μmoles (10 μc of ^{14}C) of [^{14}C]acetate/3 ml. were employed. At 122 min. 4 μmoles of DPN/3 ml. of incubation mixture was added. The times of sampling are indicated by arrows.

The first experiment provided sufficient data for the demonstration of a stoicheiometric relationship between [^{14}C]acetate incorporation and DPNH utilization in fatty acid synthesis (Table 7). This was done by comparing the DPNH utilization observed in the spectrophotometer with the expected DPNH utilization calculated from the μmoles of radioactive fatty acyls found on the chromatograms. The agreement obtained was very satisfactory in view of the fact that the data for β-hydroxybutyryl were supplied from two separate experiments, that an error of 20 % is involved in the determination of the amounts of fatty acyls present, and that the utilization of DPNH, although predominantly related to β-hydroxybutyryl formation, is also complicated by side reactions described elsewhere in the text.

The second large-scale experiment was similar to the first, except that after the steady state had become established for 70 min. 4 μmoles of DPN/3 ml. of reaction mixture was added in an attempt to reverse the reaction in the direction of fatty acid oxidation. The expected reduction of DPN occurred, and 0·182 μmole of DPNH had been formed in 9 min. (cf. Fig. 6) when the last sample was taken. In this experiment chromatograms were developed with both octanol–formic acid and butanol–acetic acid solvent systems. After scanning, the paper strips were cut into sections, and the radioactivity of the spots was determined by direct counting. The progress of this experiment is shown in Fig. 6. As in general the results from this experiment resembled both quantitatively and qualitatively those of the first experiment, only the additional information gained will be discussed. All fractions increased steadily up to the time of the steady state (44 min.) except acetohydroxamate, which reached its maximum value in 5 min. from the start. The amount of acetoacetyl was always about three times greater than that of β-hydroxybutyryl (Table 8). During the steady-state period, when no DPNH utilization was observed in the spectrophotometer, the amount of β-hydroxybutyryl increased nearly twofold and this was accompanied by more than a twofold increase in acetoacetyl. Similar increases in the saturated fatty acids also occurred during this period. Up to this point this experiment correlated well with the experiment of Fig. 5. Even when the apparent utilization of DPNH ceased, β-hydroxybutyrate continued to be synthesized. This occurred

Table 8. *Amounts of acetoacetyl–CoA and β-hydroxybutyryl–CoA formed in FASF from* [^{14}C]*acetate under standard conditions of fatty acid synthesis*

Results were obtained from radioactive counts made on paper chromatograms of the hydroxamates developed in the butanol–acetic acid–water system. The progress of DPNH utilization in this expt. is shown in Fig. 6. At 122 min. 4 μmoles of DPN/3 ml. of incubation mixture were added.

Time of sampling after start of reaction (min.) ...	4	16	44	113	131
	Total counts/min.				
Acetoacetyl	3 100	8 920	12 870	27 460	46 630
β-Hydroxybutyryl	1 080	3 240	4 240	7 250	13 280

presumably at the expense of re-reduction of DPN by some oxidizable substrate which, as is discussed below, we believe to be AMP.

A surprising and perhaps significant finding was that the re-reduction of the added DPN was not associated with the oxidation of fatty acids, as was expected, but with a very great increase in fatty acid synthesis. The increases in the amounts of β-hydroxybutyryl and acetoacetyl (cf. Table 8) were paralleled by a rise of butyryl from 2130 (0·02 μmole of acetate incorporated) to 3360 (0·032 μmole) counts/min. and of hexanoyl + higher fatty acyls from 990 (0·01 μmole) to 9060 (0·087 μmole) counts/min. This may be compared with the experiment of Fig. 5, where during the first 15 min. of synthesis in the presence of 0·4 μmole of DPNH, 0·04 μmole of acetate was incorporated into fatty acids; or with the first experiment described in this section, where the addition of 0·4 μmole of DPNH resulted in the synthesis of a further 0·035 μmole of saturated fatty acids. Evidently the reduction of DPN did not occur at the expense of fatty acyls, or of β-hydroxybutyryl, but at the expense of some other substrate, now thought to be AMP. This oxidation of AMP was associated with a marked increase in the synthesis of certain saturated fatty acids.

Chain length of fatty acids synthesized by the FASF

The paper chromatograms of the acylhydroxamates, as found in several experiments, indicated that our preparations must have synthesized butyric, hexanoic and octanoic acids at least. Since the solvent systems used for these chromatograms could not resolve acid hydroxamates with a chain length greater than C_8, the fatty acids extracted from the incubations were fractionated by column chromatography according to the reversed-phase technique of Howard & Martin (1950).

From an experiment similar in every respect to that shown in Fig. 5, the fatty acids were extracted after various intervals of incubation and resolved by reversed-phase partition chromatography. In Table 9 the results obtained on one sample, drawn from a bulk incubation 45 min. after addition of acetate, are shown. At this time (steady state) 0·181 μmole of DPNH had been utilized and 0·058 μmole of acetate incorporated into fatty acids. The FASF can effect the synthesis of fatty acids of all chain lengths up to C_{18}, but is most efficient for the short-chain acids.

Table 9. *Chain-length specificity of FASF as measured by incorporation of* [^{14}C]*acetate into various fatty acids*

Standard conditions of assay were employed with 0·4 μmole of DPNH and 10 mg. of undialysed FASF/3 ml. Incubation at room temperature for 45 min. For the extraction of labelled fatty acids, triglyceride fat obtained from mammary gland and containing 25 mg. of mixed acids was added to the sample before saponification.

Fatty acid chain length	Specific activity (counts/min. at infinite thickness of 2 cm.^{2} sample)	Fatty acid (%, w/w, of total)	Radioactivity of fatty acid (% of total)
$C_4 + C_6$	3731	7·9	46·50
C_8	1111	25·8	45·20
C_{10}	101	15·7	2·5
C_{12}	178	7·3	2·05
C_{14}*	70	6·7	0·74
C_{16}*	31	27·5	1·35
C_{18}	116	9·1	1·66

* The tetradecanoic acid sample contained also hexadec-9-enoic and the hexadecanoic some octadec-9-enoic acid.

Relative roles of DPN and TPN in fatty acid synthesis by the FASF as revealed by chromatography

The results in Table 4 gave no indication that TPN might play a specific role in fatty acid synthesis by the mammary-gland enzymes. Since, however, these conclusions were based on incorporation of acetate into total fatty acids, the possibility that TPN may cause a shift of synthesis from shorter to longer acids, without necessarily affecting the sum total of synthesis, had to be considered. Hydroxamates were prepared from all samples of the three experiments shown in Table 4 and chromatographed. The incubations in these experiments, however, were done at 38°, a condition which, as we found later, leads to significant deacylation of at least crotonyl–CoA and possibly of other acyl–CoA

derivatives also. The relative proportions of butyryl and crotonyl as shown by the paper chromatograms of the hydroxamates do not reflect, therefore, the total amounts of these acids formed in these experiments. The products split off from CoA by deacylases would accumulate in the incubation mixture and would not form hydroxamates, but would be extracted after the usual saponification into total fatty acids.

As the significance and extent of deacylase action in the FASF at 38° was appreciated, we realized that by the procedures used by us in extracting fatty acids and purifying these from contaminating [^{14}C]acetate by chromatography (cf. Popják & Tietz, 1954) our total fatty acid samples obtained from incubations made at 38° could contain a far higher proportion of crotonic acid than samples obtained from incubations at room temperature. By the usual methods of liquid–liquid partition chromatography it is doubtful if crotonic acid could be separated from other short-chain fatty acids; but we found that the vapour-phase chromatographic technique of James & Martin (1952) gave an excellent separation of crotonic acid from butyric and hexanoic acids provided that the development was made with a slow gas flow through the column (10–15 ml. of N_2/min. with a 4 ft. column at 137°).

An experiment was therefore carried out at 38° under standard conditions of fatty acid synthesis with DPNH, TPNH or their combination as hydrogen donors. At the end of incubation 20 mg. of a mixture of butyric, crotonic, hexanoic and octanoic acids was added to the flasks and the whole saponified. After extraction of fatty acids and usual purification from contaminating [^{14}C]acetate the four short-chain acids were resolved by vapour-phase chromatography; acids higher than octanoic were not analysed. Although the results shown in Table 10 revealed a surprisingly large amount of labelled crotonate when DPNH or DPNH + TPNH was employed, they gave no support to the contention that TPNH in any way enhanced the reduction of unsaturated to the saturated acid. In fact the amounts of the saturated acids formed when both nucleotides were present were less than with DPNH alone. The synthesis observed with TPNH alone can be accounted for by the slow formation of small amounts of DPNH from TPNH in the FASF. The crotonate:butyrate ratio with TPNH alone was very similar to that found in another experiment (not reported here) with DPNH alone at 6·5 min. after the addition of acetate.

Table 10. *Synthesis of short-chain fatty acids from* [^{14}C]*acetate by the FASF. Test for specificity of pyridine nucleotides*

Standard conditions of assay with 10 μmoles of potassium acetate, 0·8 μmole of DPNH or TPNH and 10 mg. of dialysed FASF in a final vol. of 3 ml. Incubation at 38° for 45 min. Fatty acid synthesis expressed in terms of acetate μmoles incorporated per flask.

Fatty acid	Fatty acid synthesis in the presence of: DPNH	TPNH*	DPNH + TPNH*
Crotonic	81·00	13·80	125·5
Butyric	8·53	3·24	5·3
Hexanoic	8·06	2·90	7·6
Octanoic	4·80	3·55	3·7

* Generated from TPN with glucose 6-phosphate (2 μmoles).

Experiments designed to elucidate further the relationship between DPNH and TPNH in fatty acid synthesis

TPNH in the spectrophotometric system. In some experiments TPNH instead of DPNH was employed in the spectrophotometric assay system. Use was made of the presence of TPN-specific glucose 6-phosphate dehydrogenase in the FASF to generate the reduced coenzyme from added TPN and glucose 6-phosphate. The failure to demonstrate a specific effect of TPNH on fatty acid synthesis from [^{14}C]acetate (cf. Tables 4 and 10) was duplicated in a failure to demonstrate an oxidation of TPNH in the FASF on addition of acetate in the presence of CoA, cysteine, ATP and Mg^{2+} ions, i.e. under conditions in which DPNH was readily utilized. A typical experiment is shown in Fig. 7, curve *A*, which records the instantaneous and stoicheiometric formation of 0·3 μmole of TPNH by the FASF on addition of 0·3 μmole of glucose 6-phosphate. The rate of the slow and apparently non-specific utilization of TPNH (0·01–0·03 μmole/hr./10 mg. of protein at 38°) in the absence of added substrate was only slightly increased by the introduction of acetate and could be accounted for by the formation of DPNH from TPNH (see later). The disappearance of not more than 0·016 μmole of TPNH could be attributed to acetate in 28 min. The addition of oxidized glutathione caused, however, a rapid oxidation of TPNH by the TPNH-specific glutathione reductase (Mapson & Goddard, 1951) also present in the FASF. This and other similar experiments indicated that TPNH, under the conditions of fatty acid synthesis, did not undergo significant destruction.

TPN, however, in the absence of ATP is destroyed by the FASF (particularly at 38°), and is only partially reduced by glucose 6-phosphate to a substance which on continued incubation ceases to react enzymically either as TPNH or DPNH (Fig. 7, curve *B*). At room temperature this destruction is much less marked (cf. Fig. 8).

Transhydrogenase. The reduction of TPN by DPNH is known to occur in mammalian tissues

(Kaplan *et al.* 1953). An attempt was made to demonstrate the presence of such a transhydrogenase in the FASF in experiments based on those of Kaplan *et al.* (1953). Three of the type shown in Fig. 8 were performed in which we made use of the TPN-specific glucose 6-phosphate dehydrogenase and TPNH–glutathione reductase present in the FASF. Two cuvettes contained the complete system with TPN and DPNH, whereas TPN was omitted from the third cell. After 20 min. GSSG was added to test for TPNH; this time was sufficient in the experiments of Kaplan *et al.* (1953) to allow for the reduction of about 0·35 μmole of TPN by DPNH. Fig. 8 shows that the rate of fall of optical density after the addition of GSSG was the same in the complete system containing TPN and DPNH as in the control without TPN. At the end of a second 20 min. period glucose 6-phosphate was added to one of the cells containing the complete system and to which GSSG had not previously been added. A rise in optical density immediately occurred, corresponding to the formation of 0·13 μmole of TPNH (Fig. 8, curve 3). When there was no further rise in optical density the addition of GSSG caused an immediate fall in optical density which was 8·5 times more rapid during the next 10 min. than the corresponding rate in the control cells. The rate fell off with time and finally became the same as that in the controls. This experiment demonstrated that there was not sufficient transhydrogenase present in the FASF to generate TPNH in detectable amounts (at least 0·02 μmole in our assay system).

Destruction and interconversion of pyridine nucleotides. In these studies DPN, DPNH, TPN and TPNH (0·2–0·8 μmole) were incubated with the FASF in the presence and absence of ATP and other cofactors of fatty acid synthesis and then tested for all four nucleotides. The destruction of TPN in the absence of ATP has already been referred to (cf. Fig. 7, curve *B*). A similar destruction also of DPN by the FASF occurred when ATP was omitted from the incubation mixture; nicotinamide, in the amounts used by Kaplan *et al.* (1953), did not protect DPN. Although ATP protected both nucleotides against destruction, no TPN was formed when DPN and ATP were incubated together. TPNH, prepared either by chemical reduction of TPN or generated at the beginning of the experiment from TPN by glucose 6-phosphate

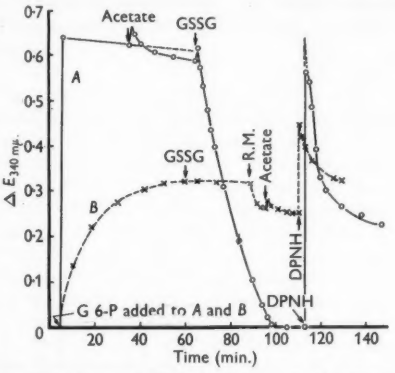

Fig. 7. Reduction of TPN by glucose 6-phosphate dehydrogenase in the FASF as measured by u.v. light absorption at 340 mμ. Curve *A*, the cuvette contained initially: CoA, 2 μmoles; cysteine, 30 μmoles; ATP, 30 μmoles; Mg^{2+}, 20 μmoles; TPN, 0·38 μmole; tris buffer, pH 9, 100 μmoles; protein (undialysed FASF) 10 mg.; total vol. 2·85 ml.; temp. 38°. Additions were made at arrows; glucose 6-phosphate (G 6-P), 0·3 μmole in 0·075 ml.; potassium acetate, 50 μmoles in 0·1 ml.; oxidized glutathione (GSSG), 1·4 μmoles in 0·05 ml.; DPNH, 0·3 μmole in 0·1 ml. Dotted straight line after 35 min. gives the extrapolated rate of TPNH disappearance which would have been observed if acetate had not been added; the dotted spike after 110 min. indicates the theoretical level of optical density due to addition of DPNH. Curve *B*, the cuvette contained in a vol. of 2·85 ml. initially only Mg^{2+}, TPN, buffer and protein in the same amounts as for curve *A*. Additions were: G 6-P, 0·6 μmole in 0·15 ml.: GSSG, 1·4 μmoles; reaction mixture added at R.M. consisted of CoA, 2 μmoles, cysteine, 30 μmoles and ATP 30 μmoles in 0·65 ml.; potassium acetate, 50 μmoles; DPNH, 0·1 μmole in 0·03 ml. Temp. 38°.

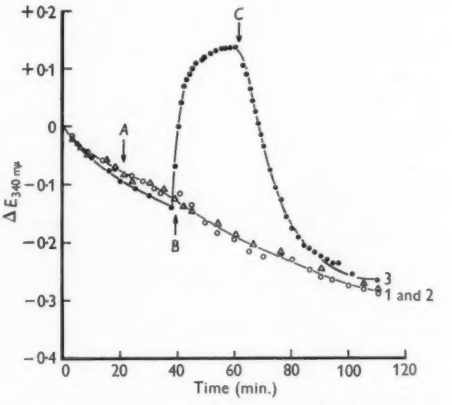

Fig. 8. Test for the transhydrogenase reaction in the FASF. The complete system contained 0·4 μmole of DPNH, 0·25 μmole of TPN, 8·3 mg. of FASF (dialysed), 230 μmoles of potassium phosphate buffer, pH 7·5, and 100 μmoles of nicotinamide, in a final vol. of 3 ml. Room temperature (1) ○, DPNH + TPN; (2) △, DPNH, no TPN; (3) ●, DPNH + TPN. At *A*, 5·6 μmoles of GSSG were added to 1 and 2; at *B*, 0·18 μmole of glucose 6-phosphate was added to 3; at *C*, 5·6 μmoles of GSSG were added to 3.

dehydrogenase, showed much greater stability than TPN on incubation with FASF. However, small quantities of DPNH were always formed (up to 0·05 μmole) from TPNH in the presence or absence of ATP.

Dephosphorylation reactions. In a number of experiments the liberation of orthophosphate from ATP, ADP, AMP, TPN, TPNH and from inorganic pyrophosphate in the FASF was measured under conditions similar to those used in the study of fatty acid synthesis. It was found that orthophosphate was liberated from the three adenine nucleotides at about the same rate (about 1 μmole/10 mg. of protein/30 min. at 38° from 30 μmoles of the nucleotides), and that this rate did not vary greatly from one FASF to another. The rate of liberation of orthophosphate was linear with time up to 120 min. Sodium pyrophosphate was hydrolysed 20–30 times as fast as were the adenine nucleotides. Such rapid liberation of orthophosphate was also observed when ATP was incubated with acetate, CoA and cysteine, and was presumably due to the hydrolysis of inorganic pyrophosphate generated from ATP during the activation of acetate. The amounts of orthophosphate appearing in such experiments were no greater than could be accounted for by the stoicheiometry of the acetate activation reaction plus the slow non-specific hydrolysis of ATP, AMP and of CoA. CoA, TPN and TPNH were also hydrolysed by the FASF, but at a rate not greater than that for adenine nucleotides

Reduction of DPN by AMP. Popják & Tietz (1955) reported that the fatty acid-synthesizing soluble enzyme preparations of mammary gland consumed oxygen in the presence of acetate and that this oxygen consumption depended on the amount of ATP added. There is now good evidence indicating that this oxygen consumption is due to an 'AMP-oxidase' that is probably linked with a xanthine oxidase system, both present in the FASF.

When DPN and AMP, in amounts equivalent to those arising in our system during the course of fatty acid synthesis, were incubated together with dialysed or undialysed FASF, reduction of DPN occurred (Fig. 9), the rate and extent of which depended upon the temperature at which the experiment was carried out. At room temperature reduction of DPN did not occur for about 1 hr. If the reaction mixture was first incubated at 38° for 5 min. with either the AMP or the FASF omitted, and the reaction then initiated by the addition of the missing component, the reduction of DPN commenced after about 15 min. The reduction was more rapid if the mixture was left at 38° than it was at room temperature, but in either case the 'lag period' was shorter than if the reaction mixture was left at room temperature throughout. Hypoxanthine reduced DPN more rapidly at room temperature without showing a lag period, ATP and ADP reduced DPN at one-eighth and one-half of the rate obtained with AMP.

It was thought possible that during the lag phase AMP was degraded to hypoxanthine and that this was oxidized by a xanthine oxidase-type enzyme. The presence of such an enzyme in the FASF has, in fact, been demonstrated in a number of experiments in which hypoxanthine or xanthine was used as substrate and potassium ferricyanide, 2:6-dichlorophenolindophenol or oxygen was used as electron acceptor.

The part played by the reduction of DPN by AMP in fatty acid synthesis was tested in the following experiments. The reduction of DPN by this reaction was inhibited by the xanthine oxidase inhibitors 2:5:6-triamino-4-styrylpyrimidine (CB 1019), phenylpurine and pteridine aldehyde (2-amino-6-formyl-4-hydroxypteridine) (Bergel, 1956). These substances caused considerable inhibition of the reduction of DPN by AMP during the first

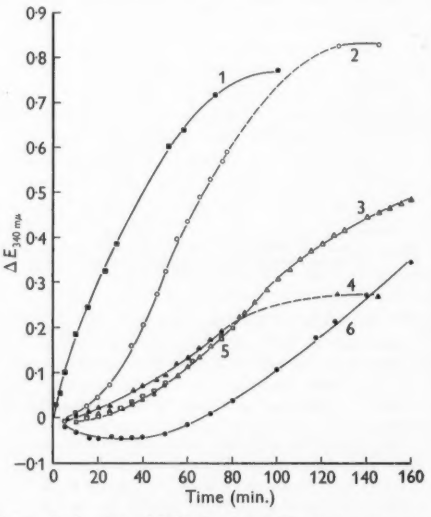

Fig. 9. Reduction of DPN by AMP. The test system contained 2 μmoles of AMP, 0·4 μmole of DPN, 100 μmoles of tris buffer, pH 9, 20 μmoles of $MgCl_2$ and 8·3 mg. of FASF (dialysed) in a final vol. of 3 ml. (1) ■, Hypoxanthine (2 μmoles), instead of AMP, at room temperature throughout; (2) ○, preliminary incubation at 38°, then read at 38° in an electrically heated cell housing; (3) △, preliminary incubation at 38°, then read at room temperature; (4) ▲, as (2) without AMP; (5) □, as (2) with $3·4 \times 10^{-5}$ M CB 1019. Similar curves were obtained with $6·8 \times 10^{-5}$ M pteridine aldehyde and with $6·8 \times 10^{-5}$ M phenyl purine; (6) ●, read at room temperature throughout, no preliminary incubation at 38°.

Table 11. *Effects of xanthine oxidase inhibitors and of continuous reduction of DPN by alcohol dehydrogenase on fatty acid synthesis from* [^{14}C]*acetate*

Standard conditions of assay were used with 0·8 μmole of DPNH or TPN, 10 μmoles (10 μc of ^{14}C) of potassium acetate and 5 mg. of dialysed FASF in a final vol. of 3 ml. The additions were made at the beginning in final concentrations and amounts indicated. The fatty acid synthesis was started by introduction of acetate. Expts. 1 and 2 were carried out 3 weeks apart but with the same FASF preparation. Incubations were at 38° for 30 min. Results expressed as μmole of acetate incorporated into fatty acids per assay.

Pyridine nucleotide used and other additions	Expt. 1	Expt. 2
Control: no DPNH or TPNH	0·005	0·002
DPNH	0·136	0·147
TPNH*	—	0·008
DPNH + TPNH*	—	0·115
DPNH + alcohol dehydrogenase (0·3 mg.) + ethanol (10 μmoles)	—	0·273
DPNH + lipoic acid (2 μmoles)	—	0·138
DPNH + phenyl purine ($6·8 \times 10^{-5}$ M)	0·063	—
DPNH + 2:5:6-triamino-4-styrylpyrimidine ($3·4 \times 10^{-5}$ M)	0·159	—
DPNH + pteridine aldehyde ($6·8 \times 10^{-5}$ M)	0·136	—
DPNH + hypoxanthine (2 μmoles)	0·214	—
DPNH + hypoxanthine + 2:5:6-triamino-4-styrylpyrimidine	0·134	—
DPNH + hypoxanthine + pteridine aldehyde	0·184	—

* TPN + glucose 6-phosphate (2 μmoles).

30 min. of the reaction (cf. Fig. 9). Their effect upon the utilization of DPNH in the fatty acid-synthesizing system, at the concentrations given in the legend to Fig. 9, was variable. CB 1019 was without effect, pteridine aldehyde increased the utilization of DPNH by 30 %, whereas phenyl purine depressed the utilization to 25 % of the control value. These findings were reflected in an experiment in which the effect of the inhibitors upon [^{14}C]acetate incorporation into fatty acids was tested (Table 11). CB 1019 and pteridine aldehyde were without effect on incorporation of [^{14}C]acetate, whereas phenyl purine depressed it to one-half of the control value. Hypoxanthine increased incorporation by 55 %, but the addition of inhibitors with the hypoxanthine reduced the incorporation, in one instance to the control level. Alcohol dehydrogenase, although far more effective in keeping DPN fully reduced in our experiments than hypoxanthine, stimulated fatty acid synthesis only a little more than the purine.

DISCUSSION

We think that the most significant part of our results is the demonstration for the first time of a fatty acid cycle (or spiral) in an organized enzyme system as postulated by Lynen & Ochoa (1953) and Lynen (1954). The same concept of synthesis of fatty acids by a stepwise lengthening of fatty acid chains by the addition of C_2 units to shorter chains has been expressed earlier (Popják, French, Hunter & Martin, 1951; Popják, 1952) on the basis of experiments on whole animals, although the nature of intermediates could not be defined. The mechanism of fatty acid synthesis according to these ideas is a reversal of the β-oxidation of fatty acids already achieved with purified enzyme preparations (cf. Lynen, 1954; Green, 1954). We may state now with some confidence that the steps involved in the synthesis of butyric acid involve the reduction of acetoacetyl–CoA to β-hydroxybutyryl–CoA, the dehydration of the latter to crotonyl–CoA, followed by a second step of reduction. The presumption is that a similar mechanism operates in the synthesis of higher acids also. In our enzyme preparations DPNH can act as the electron donor for both reductive steps. The first reductive reaction catalysed by the β-hydroxybutyryl dehydrogenase is a well-understood reaction (Wakil *et al.* 1954; Lynen *et al.* 1952), dependent on the participation of DPNH. However, the reduction of crotonyl–CoA with a natural electron donor has so far defied the efforts of those laboratories working with purified enzymes. Stansley & Beinert (1953) only succeeded in reducing crotonyl–CoA to butyryl–CoA with reduced benzyl viologen, which was itself kept reduced with xanthine oxidase. In their system no fatty acid higher than butyric was formed.

The report by Langdon (1955) that crude liver preparations will reduce crotonyl–CoA with TPNH made us concentrate our efforts particularly on this possibility, but all our attempts to demonstrate a specific role of TPNH in the synthesis of *n*-fatty acids containing an even number of carbons in their chain met with complete failure. In the absence of a demonstrable transhydrogenase reaction in the FASF, TPNH could not have been formed from DPNH, even if the FASF had contained catalytic

amounts of TPN. Our failure to demonstrate a significant effect of TPN or TPNH on fatty acid synthesis could not be attributed to the destruction of these coenzymes by the FASF as there was but a slight loss of TPN or TPNH in the FASF under conditions of fatty acid synthesis. The slight fatty acid synthesis observable when TPNH alone was used as electron donor could be attributed to small amounts of DPNH formed in our preparation. All these negative findings may be contrasted with the very positive effects of DPNH. The stoicheiometric relationship established between acetate-dependent DPNH utilization and acetate incorporation into fatty acids and their intermediates gave added support to our conclusion that DPNH acts as the primary electron donor in the reaction catalysed by both the β-hydroxyacyl dehydrogenase and fatty acyl dehydrogenase. Any attempt to establish a stoicheiometric relationship of this kind, in a complex series of reactions, with a partially purified enzyme preparation in which side reactions (e.g. the reduction of DPN by AMP or deacylation reactions) can take place, is certain to present difficulties; and if a correlation between DPNH utilization and [^{14}C]acetate incorporation can be shown to within 50 % something will have been achieved. In fact, most of the correlations obtained were considerably better than this (cf. Table 7). Such a satisfactory relationship could only be demonstrated in experiments carried out at room temperature, since at 38° interference from side reactions became so marked that the quantitative assessment of results became impossible.

At first we thought that the metabolism of AMP in the FASF, whereby DPN is reduced at its expense, does no more than provide a supplementary source of electrons for the reductive steps of synthesis. However, in experiments of long duration (over 50 min. of incubation), such as is partly shown in Fig. 6, and when the AMP-oxidase may be expected to come into full play, a degree of stimulation of fatty acid synthesis is observed far in excess of that attributable merely to generation of additional amounts of DPNH. This focused our attention on possible substances derived from AMP and which might exert a specific catalytic role in the second reductive step of fatty acid synthesis. Among these hypoxanthine was excluded by the experiments shown in Table 11, as it was found that this substance did not stimulate fatty acid synthesis any more than did alcohol dehydrogenase + ethanol. Preliminary experiments, however, strongly suggest that inosine monophosphate, derived from AMP by hydrolytic deamination, in the presence of DPNH catalyses, in an as yet undefined manner, the reduction of crotonyl–CoA to butyryl–CoA by the FASF. If further experiments confirm these results a good teleological reason may be provided for the splitting of the pyrophosphoryl from ATP during the acetate-activation reaction, since the metabolism of the resulting AMP would provide a catalyst for subsequent reactions.

SUMMARY

1. A partially purified enzyme preparation has been obtained from lactating rabbit mammary gland that catalyses the synthesis of even-numbered-chain fatty acids from C_4 to C_{18}, with the shorter-chain fatty acids preponderating.

2. This synthesis is achieved from acetate, coenzyme A (CoA), adenosine triphosphate and stoicheiometric amounts of reduced diphosphopyridine nucleotide (DPNH). The intermediates involved have been studied by paper chromatography, as their hydroxamates. The findings strongly suggest that fatty acid synthesis in these enzyme preparations takes place by a stepwise condensation of C_2 units, through the reversal of the now well-established β-oxidation of even-numbered-chain fatty acids.

3. TPNH and lipoic acid in our system have no stimulatory effect upon the synthesis of even-numbered-chain fatty acids by the mechanism outlined above.

4. DPNH appears to act as a specific electron donor for both the reduction of β-ketoacyl–CoA to β-hydroxyacyl–CoA and of unsaturated fatty acyl–CoA to saturated fatty acyl–CoA. This latter reduction step appears to be stimulated in some manner, as yet undefined, by a metabolic derivative of adenosine 5-phosphate, which is not hypoxanthine, and which may possibly be inosine monophosphate.

The authors wish to thank Dr David E. Green for a generous gift of coenzyme A, and Professor F. Bergel and Professor A. Albert for gifts of xanthine oxidase inhibitors. Grateful acknowledgement is made to the Eli Lilly Foundation for a grant to the Medical Research Council for the purchase of our Spinco model L preparative ultracentrifuge.

Mr R. Hancock participated in the development of certain of the chromatographic methods. It is a pleasure to acknowledge the skilled technical assistance of Miss Mary O'Donnell and Mr Barry Buckingham and the help of Miss J. Ashenden in the preparation of the diagrams. Fig. 5 is reproduced from Hele & Popják (1955) by permission of Elsevier Publishing Co.

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The Amino Acid Composition of Fish Collagen and Gelatin

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The major protein constituent of the skin, bones, swim bladder and scales of fishes resembles in many ways the more widely studied collagen of mammals. Thus it contains hydroxyproline and hydroxylysine, shows striated fibrils with the electron microscope (Borasky, 1950; Schmitt, Gross & Highberger, 1955; Damodaran, Sivaraman & Dhavalikar, 1956), and has characteristic wide-angle and narrow-angle X-ray diffraction patterns (Bear, 1952).

Amino acid analyses of fish collagens have been reported by Beveridge & Lucas (1944), who used mainly gravimetric methods, for isinglass from the swim bladder of hake (*Urophycis*); by Block, Horwith & Bolling (1949) for scales of herring (*Clupea*) and by Neuman (1949), who used microbiological-assay techniques, for halibut skin and for gelatin prepared from the scales of an unspecified fish. The recent study on the composition of elastoidin from the fin rays of the shark (*Carcharinus melanopterus*) by Damodaran *et al.* (1956), who used resin chromatography, has confirmed the results of wide-angle X-ray diffraction work (Astbury, 1938) in placing this unusual protein within the collagen group.

Interest in fish collagens has recently been stimulated by the suggestion that their reduced structural stability compared with mammalian collagens (shown by a lower range of shrinkage temperatures), is related to a lower content of hydroxyproline (Gustavson, 1953). Interchain hydrogen bonding between hydroxyl groups of hydroxyproline and backbone carbonyl groups was, on this basis, suggested as an important stabilizing feature of the collagen structure (Gustavson, 1955). This hypothesis has since been applied, without further proof, in interpreting details of the structure of collagen at two levels of size, in connexion with X-ray diffraction (Ramachandran, 1956) and electron-microscope techniques (Reed, Wood & Keech, 1956) respectively. A summary of recent work on the connective tissues of fishes is given by Hamoir (1955).

The present investigation provides information about the composition of collagen from a few species representing a zoologically wide range of fish types, members of all three surviving classes, Elasmobranchii, Actinopterygii and Crossopterygii (Young, 1950; Trewavas, White, Marshall & Tucker, 1955) being included. The experimental methods were

identical with those used in an earlier study of mammalian collagens (Eastoe, 1955), so that valid comparisons of small differences in composition can be made.

EXPERIMENTAL

Materials

Sturgeon swim-bladder collagen. Commercially available isinglass from the swim bladder of the Beluga sturgeon (*Acipenser huso huso*) was used as received. It is not known what earlier treatment this relatively pure product had undergone. The isinglass gave a 95% yield of clear gelatin when extracted under the conditions stated in Table 1.

Cod-bone gelatin. Cod (*Gadus morrhua*) vertebrae, which had been preserved in ice, were placed in 8% HCl for 3 days. The adhering muscle was pulled off and the demineralized bone was transferred to 10% acetic acid for 14 days. The bone collagen was repeatedly washed to remove acid and converted into gelatin by hot-water extraction (Table 1).

Shark-skin gelatin. Shark (probably *Selachus maximus*) skin was demineralized in 5% HCl for 5 days, cut into pieces and treated with saturated SO_2 solution for 20 days at 2°. Gelatin was extracted under the conditions given in Table 1.

Lung-fish collagen and gelatin. Skin of the Australian lung fish (*Neoceratodus*), which had been preserved by salting and drying, was soaked in water and the outer pigmented layer pulled off and discarded. The skin was cut into pieces, soaked in two changes of 10% NaCl for 24 hr. each, washed for 48 hr. in eight changes of water, dehydrated in acetone and ether, and finally air-dried. Moisture and ash contents of this and other proteins actually analysed are given in Table 1.

The lung-fish skin was soaked in 0·1 N-HCl for 18 hr., the acid was then neutralized by addition of NaOH soln. and the gelatin extracted in two stages (Table 1), giving 45 and 33% recoveries of the weight of skin respectively.

Methods

Hydrolysis. The proteins were hydrolysed in approx. 100 times their weight of 20% (w/w) HCl at 100° in sealed tubes for the times stated in Table 2.

Amino acid analysis, etc. Amino acid analyses, etc. were carried out by resin chromotography (Moore & Stein, 1951) exactly as described by Eastoe (1955), and the same corrections were applied. Hydroxyproline was determined by the method of Neuman & Logan (1950).

Kinematic viscosities. Kinematic viscosities of 6·67% (w/w) aqueous solutions of the gelatins with specified moisture and ash contents (Table 1) were measured at pH 6·0–6·5 according to B.S. 757:1944.

Rigidity determinations. Rigidity determinations were carried out on 6·67% gels, matured for 17 hr. at the specified temperatures, according to the method of Saunders & Ward (1953).

Shrinkage temperatures. Shrinkage temperatures were measured by visual observation of water-saturated samples freely suspended in toluene.

RESULTS

The amino acid contents of fish collagens and gelatins calculated on the weight of dry, ash-free protein are given in Table 2. The recovery of total

Table 1. *Moisture and ash contents of collagens and gelatins analysed; extraction conditions and physical properties of the gelatins*

Material		Water* (%)	Ash* (%)	Pretreatment (see text)	Extraction conditions			Kinematic viscosity (centistokes at 40°)	Rigidity† (dynes/cm.²)
					Time (hr.)	Temp.	pH		
Sturgeon swim bladder	Collagen	17·1	0·3	—	—	—	—	—	—
	Gelatin	—	—	None	3·0	60°	4·0	5·8	59 000
Cod bone	Gelatin	11·3	1·3	Acetic acid	3·0	60	4·4	5·3	28 000 at 0° 300 at 10° 0 at 14°
	Gelatin	—	—	Acetic acid	2·0	80	4·2	4·5	18 100 at 0° 0 at 10°
Shark skin	Gelatin	—	—	HCl, SO_2	3·0	60	4·0	4·3	45 000
	Gelatin	13·3	2·8	HCl, SO_2	3·0	80	4·0	2·5	14 000
Lung-fish skin	Collagen	18·3	0·14	—	—	—	—	—	—
	Gelatin	14·1	5·0	HCl	5·5	65	5·5	‡	56 000
	Gelatin	—	—	HCl	5·5	80	3·5	‡	8 400

* Moisture and ash values are given only for those proteins for which the amino acid analyses are reported.
† At 10° unless otherwise specified.
‡ Not determined.

nitrogen ranged from 97·6 to 102·5 %, the extreme values not greatly exceeding the expected experimental error of ±2 %. The recovery by weight ranged from 93·7 % for collagen of lung-fish skin to 99·2 % for sturgeon swim bladder. These figures were lower than those for mammalian materials, and suggested a higher content of non-nitrogenous components in some of the fish proteins.

Traces of cysteic acid and possibly cystine were detected in the hydrolysates but amounts were such as to suggest that the cystine content did not exceed 0·1 %. Tryptophan was not determined.

Some physical properties of the gelatins derived from fish collagens are summarized in Table 1. The viscosity values are only slightly below those of the mammalian gelatins investigated previously (Eastoe, 1955). This suggests that the fish and mammalian gelatins are of the same order of average molecular size.

The rigidities of the sturgeon, shark and lung-fish gelatins were somewhat lower than those of mammalian gelatins. It is possible that these reduced values arose through failure to find the optimum conditions for extraction of gelatin from the fish materials, although it is thought that there was probably a definite intrinsic difference. Pretreatment and extraction conditions had been adjusted for each material in an attempt to produce gelatin with minimum degradation, but the effect of the various variables was not known with the same certainty as for mammalian gelatin, the extraction of which has been widely studied.

The rigidity of the material extracted from cod bone was markedly lower than for the other fish gelatins. At 10° a 6·67 % gel gave a low value, so that the measurement was repeated at 0°. The melting point of this gel was 12°, compared with 30° for mammalian gelatin. Gelatins from cod skin and swim bladder have also been found to have comparable, greatly diminished gelling properties, 6·67 % gels melting at 13° (Dr A. Courts, private communication).

Table 2. *Amino acid composition of fish collagens and gelatins*

Values are given as g. of amino acid/100 g. (*G*), and moles of amino acid/10^5 g. (*M*) of dry ash-free protein.

	Sturgeon swim-bladder collagen		Cod-bone gelatin		Shark-skin gelatin		Lung-fish skin			
							Collagen		Gelatin	
Duration of hydrolysis (hr.) ...	24		24		24		48		48	
	G	*M*	*G*	*M*	*G*	*M*	*G*	*M*	*G*	*M*
Alanine	11·6	130	10·4	115	11·2	126	11·7	131	11·9	134
Glycine	27·7	369	28·2	376	26·5	353	24·0	319	26·1	348
Valine	2·31	19·7	2·32	19·7	2·71	23·2	2·56	21·8	2·21	18·9
Leucine	2·55	19·4	3·26	24·8	3·32	25·3	3·37	25·8	2·75	21·0
Isoleucine	1·65	12·5	1·64	12·5	2·71	20·6	1·64	12·5	1·34	10·2
Proline	12·8	111·7	12·4	107·8	13·9	120·2	14·8	129·0	15·8	137·2
Phenylalanine	2·53	15·4	2·04	12·3	2·43	14·7	2·60	15·7	2·41	14·6
Tyrosine	0·46	2·6	0·63	3·6	0·26	1·5	0·19	1·1	0·14	0·8
Serine*	5·8	55·2	7·9	75·3	5·0	47·2	4·71	44·8	4·71	44·8
Threonine*	3·79	31·9	3·22	25·7	3·24	27·3	3·18	26·7	3·01	25·2
Cystine†	—	—	—	—	—	—	—	—	—	—
Methionine‡	1·43	9·6	2·26	15·1	1·59	10·6	0·59	4·1	0·54	3·6
Arginine	10·0	57·3	9·1	52·3	9·3	53·3	9·1	52·2	9·9	56·9
Histidine	0·83	5·3	1·24	8·0	1·26	7·8	0·80	5·2	0·75	4·8
Lysine	3·46	23·8	3·66	25·1	3·76	25·7	3·63	24·8	3·63	25·0
Aspartic acid	6·9	51·9	7·5	55·9	6·0	45·1	6·6	49·8	6·2	46·7
Glutamic acid	11·4	77·1	11·4	77·7	10·3	69·7	11·9	80·8	11·9	81·3
Hydroxyproline	11·8	89·6	8·3	63·2	10·9	83·2	9·8	74·8	10·8	83·2
Hydroxylysine	1·90	11·7	1·42	8·8	0·82	5·0	0·88	5·4	1·08	6·7
Total	118·9	1093·7	116·9	1078·8	115·2	1059·4	112·05	1024·5	115·17	1062·9
Amide N§	0·63	44·9	0·67	48·0	0·43	31·1	0·67	47·9	0·63	45·0
Total N	18·54		18·38		18·21		18·24		18·21	
Mean wt. of residue	90·7		90·3		90·8		91·35		90·37	
Z‖	119·9		119·9		119·8		119·7		119·9	
Recovery by wt. (%)	99·2		97·5		96·2		93·7		96·1	
Recovery of N (%)	102·5		101·7		99·9		97·6		101·2	

* Corrected for decomposition during hydrolysis (see Eastoe, 1955).
† Trace (see text).
‡ Sum of methionine and methionine sulphoxide peaks.
§ Corrected for ammonia formed by decomposition of serine and threonine.
‖ Chibnall (1942).

DISCUSSION

Comparison with previously published results

A number of analyses of fish connective-tissue proteins have been made by earlier workers, but comparison is complicated by the wide range of types examined and the consequent probability of variation between collagens from zoologically remote species. In Table 3, the available data are summarized for glycine and those amino acids present in substantially different proportions in fish collagen compared with mammalian material.

Almost all of the results support earlier findings that, in general, the imino acids proline and hydroxyproline (Gustavson, 1955) are reduced in fish, compared with mammalian collagen, whereas serine (Neuman, 1949), threonine and methionine (Beveridge & Lucas, 1944) are increased in fish collagen. Large amounts of glycine are reported throughout except by Beveridge & Lucas (1944).

The hydroxyproline value obtained for halibut skin by Neuman & Logan (1950) and the present values obtained by the same method appear to be systematically higher than those of Gustavson (1955) for cod- and pike-skin collagens, which are based on Kjeldahl nitrogen determinations of hydroxyproline separated on Dowex 50 resin. This discrepancy, the reason for which is not clear, makes detailed comparison of hydroxyproline data difficult. The very low value for hydroxyproline in hake swim bladder (Beveridge & Lucas, 1944) is suspect, since it is stated by these authors to be a minimal one, possibly much below the true value. The figure for shark elastoidin was obtained by the ninhydrin method.

Characteristics of the composition of fish collagens

Fish collagens and gelatins resemble those of mammals (Eastoe, 1955) in containing the same amino acids in, broadly speaking, similar proportions. A rather wider range of composition is observed in fishes than in mammals, which is not surprising in view of the greater evolutionary age of fishes. As a group they have been exposed to wide environmental changes during the long period of existence, and their consequent adaptation has led to a great variety of types. Since some of the more primitive types have fortuitously survived relatively unchanged, alongside those more recently evolved, a wide range of material of evolutionary interest is available for biochemical work.

The diminished content of imino acids in fish collagens compared with those of mammals is well marked. Hydroxyproline, in particular, is notably low, although showing considerable variation among fish species. The lowest value in the present

Table 3. *Comparison of present values for certain amino acids with reported values*

Values refer to amino acid N as a percentage of total N.

Reference	Eastoe (1955)	Present study				Neuman (1949)	Block *et al.* (1949)	Beveridge & Lucas (1944)	Damodaran *et al.* (1956)	Gustavson (1955)	
Material	Ox-bone collagen	Sturgeon swim bladder	Cod-bone gelatin	Shark-skin gelatin	Lung-fish skin gelatin	Halibut skin	Herring scales	Hake swim bladder	Shark* elastoidin	Cod skin	Pike skin
Glycine	27·5	27·9	28·7	27·2	26·7	30·0	24·0	9·8	27·0	—	—
Proline	10·1	8·4	8·2	9·3	10·5	9·1	6·5	11·9	9·2	7·2	8·8
Hydroxyproline	8·2†	6·8†	4·8†	6·4†	6·4†	5·3†	—	2·8	5·4	3·4	4·6
Serine	2·9	4·2	5·8	3·6	3·4	4·3	6·2	3·0	2·5	—	—
Threonine	1·5	2·4	2·0	2·1	1·9	2·3	2·1	2·1	1·6	—	—
Methionine	0·4	0·7	1·2	0·8	0·3	1·2	1·7	1·4	0·96	—	—
Shrinkage temp.	65°	50°	40°	53°	63°	40° ‡	—	—	63–64°	40°	55°

* Contained 3·16% of tyrosine N. † Determined by method of Neuman & Logan (1950). ‡ Gustavson (1953).

study is 4·8 % (based on nitrogen content) for cod-bone gelatin, which may be compared with 8·2 % in ox-bone collagen.

Increased amounts of the aliphatic hydroxy amino acids serine and threonine were present in all the fish proteins examined, compared with the amounts in mammalian collagen. The hydroxylysine content was substantially increased in two of the fish materials. The increase of hydroxyl groups in the molecule, owing to the larger numbers of aliphatic hydroxy amino acid residues in fishes, was approximately equal to the loss of groups from the diminished number of hydroxyproline residues. This balancing effect results in the collagen molecule containing approximately the same proportion of hydroxyl groups in fishes and mammals.

The methionine content is very variable in fish collagens, ranging from nearly three times the mammalian figure in cod to some 60 % of it in the lung fish. Glycine and alanine, both amino acids present in large quantities in collagen, occur in almost identical amounts in fishes and mammals. In the absence of satisfactory physical criteria of purity for a fibrous protein such as fish collagen, the high values for these two amino acids suggest that the preparations were not grossly contaminated with protein impurities, which the hydroxyproline values taken alone might otherwise imply. A somewhat enhanced arginine content, offset by diminished lysine, is apparent in fish compared with mammalian collagen. The histidine contents of cod and shark gelatins are somewhat higher than the remaining fish materials, which have values closely similar to mammalian collagen. Thus the collagen of both mammals and fishes is notably low in histidine.

In all the fish collagens and acid-processed gelatins derived from them, the number of free carboxyl groups (aspartic acid + glutamic acid − total amide) is nearly equal to the sum of the basic residues (arginine + lysine + histidine), excluding hydroxylysine. It is particularly interesting that this relationship still holds for shark gelatin, where the carboxylic acids are diminished in amount compared with the other species, but this is offset by a correspondingly low amide content. The same relationship holds for mammalian collagens and may be regarded as an unusual characteristic of this group of proteins. The presence of hydroxylysine upsets this balance, resulting in a slight preponderence of basic groups in the molecule, which accounts for the iso-ionic point of acid-processed gelatins, and presumably the parent collagens, lying well to the alkaline side of neutrality (pH 9·0–9·5).

The effect of converting fish collagen into gelatin was to increase further those amino acids present in large amounts, with a corresponding reduction in those present in small quantities. These trends had already been noted in mammalian collagen (Eastoe, 1955).

The greatest departure from the mammalian composition is found in cod (*Gadus morrhua*), which is a member of the order Teleostei containing the most recently developed, specialized and currently successful of the actinopterygian fishes. The sturgeon (*Acipenser huso huso*), a comparatively unchanged member of the primitive palaeoniscid stock of the Actinopterygia, showed less extreme values for hydroxyproline, serine and threonine.

The shark, a representative of a separate class, Elasmobranchii, showed no great variation in composition from the sturgeon. The Australian lung fish (*Neoceratodus*) is particularly interesting in possessing a skin with a high shrinkage temperature, close to that of mammalian skins. The composition was fairly close to that of mammals with slightly enhanced serine and threonine and reduced hydroxyproline. The lung fish, as one of the four surviving species of the class Crossopterygii, is generally supposed to be more closely related to land vertebrates than any other living fish (Young, 1950).

Much labour would be needed for a systematic investigation of the composition of collagen in the many types of fishes; the present study gives some indication of the variations to be expected among the main groups.

Stability of intermolecular structure of collagen

Gustavson (1953, 1955) has pointed out the lower degree of intermolecular stabilization of fish (cod-skin) collagen, compared with mammalian collagen. Fish collagens are readily gelatinized, easily degraded and solubilized by acids and alkalis, and are attacked by trypsin and dissolved by anionic detergents. Mammalian collagens show much greater resistance under these conditions and have a higher shrinkage temperature. The ready conversion of cod skin and swim bladder into soluble degradation products has been confirmed in this Laboratory and the low rigidity of gelatin from cod bone (Table 1) is possibly a further manifestation of the low intermolecular stability of the parent collagen. Saunders & Ward (1955) have shown that the rigidity of different fractions of mammalian gelatin is independent of viscosity and also probably of molecular weight, above a limiting value. They have suggested that another factor, probably of a specific structural nature, controls the rigidity of gelatin gels. Ability to form extensively hydrogen-bonded systems may control both the interchain stability of collagen and the gelling of gelatin.

Gustavson (1955) has classified collagens of fish skins into two distinct groups on the basis of shrinkage temperature (T_s): (1) cold- and deep-water fishes (T_s 37–45°) and (2) warm- and surface-water fishes (T_s 50–57°). Hamoir (1955) considered this

division too rigid and suggested that there is a regular transition of shrinkage temperature from 35 to 59°. Shrinkage temperature does not appear to be related to the broad zoological classification, but rather to the habitat of a species. Thus among the Teleostei, the cod has a shrinkage temperature of 40°, whereas that of the pike (*Esox lucius*) is 55°.

Of the four species examined here, the cod alone had a shrinkage temperature near the lower end of the scale (Table 3), sturgeon and shark had much higher values, which placed them among Gustavson's pelagic fishes, and lung fish had the exceptionally high value of 63°. The marked divergence of the composition of cod gelatin from the mammalian type has already been pointed out, as well as its diminished rigidity. The remaining three fish types have hydroxyproline contents intermediate between cod and mammals, the shrinkage temperatures (with the exception of lung fish) and gel rigidities being also intermediate. Among these collagens of individual species, however, the physical properties do not run parallel to the hydroxyproline contents.

Although it seems probable that the intermolecular stability of the polypeptide chains in collagen and gelatin gels increases with hydroxyproline content, the existence of a specific hydroxyproline hydrogen bond remains somewhat speculative. It is questionable whether a single bond, having an energy of only a few kcal./mole, such as a hydrogen bond, would be of much importance acting alone in proteins where extensive backbone hydrogen bonding could occur. Hydroxyproline hydrogen bonds would have a considerable total energy for a collagen molecule of molecular weight 350 000. This quantity of energy might be important, although its very wide distribution suggests that for its effective utilization the hydroxyproline bonds would need to occupy key positions in a systematically arranged set of backbone bonds. It is also possible that other factors influence the hydrothermal stability of proteins of the collagen type. Damodaran *et al.* (1956) have pointed out the probable effect of the high contents of tyrosine and cystine on the hydrothermal properties of the rather specialized collagen in shark elastoidin. The abnormally high shrinkage temperature of lung-fish gelatin cannot be explained entirely in terms of its hydroxyproline content.

SUMMARY

1. The amino acid compositions of collagens and derived gelatins from sturgeon, cod, the shark (*Selachus maximus*) and the Australian lung fish have been determined.

2. These fish collagens show a similar amino acid distribution to mammalian collagen, with decreased amounts of proline and hydroxyproline, and increased serine, threonine and, in some cases, methionine and hydroxylysine.

3. Gelatin prepared from cod bone has a very low rigidity at 10°, whereas sturgeon, shark and lung-fish collagens give rise to gelatins having rigidities of the same order as, but probably somewhat below, those of mammalian gelatins.

4. In general, the shrinkage temperature of the collagen and gel properties of extracted gelatin decrease with decreasing hydroxyproline content. Certain departures in detail from this behaviour indicate that other unknown features of composition may influence the stability of linkages between polypeptide chains.

5. Variations in the properties and composition of fish collagens seem to be related to the water temperature of the normal habitat, rather than to considerations of broad zoological classification.

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Further Studies on Seminal Ergothioneine of the Pig

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In a previous paper (Heath, Rimington, Glover, Mann & Leone, 1953) it was concluded that the mature boar was capable of utilizing the sulphur of methionine for the synthesis of ergothioneine but not that of inorganic sulphate or thiolhistidine. Recent work by Melville, Otken & Kovalenko (1955) showed that, in the immature pig, methionine sulphur does not act as a source of ergothioneine sulphur. In order to clarify the situation it was decided to repeat the earlier experiments. The present paper reports the results of this study, together with some new observations on the incorporation of radioactive sulphur administered as [^{35}S]methionine into spermatozoa and seminal plasma of the boar.

MATERIALS AND METHODS

Experimental animal and diet. The animal used in the present study was a boar from an inbred strain of 'large whites' maintained at the Animal Research Station. The boar was 7 years' old and weighed 300 kg. It was fed twice daily, the daily ration consisting of approx. 3·5 kg. of a feed mixture containing 40% of middlings (wheat), 50% of barley meal, 12% of fish meal, 8% of lucerne meal and 2% of a mineral mixture. Regular removal of faeces was carried out and care was taken to avoid contamination of feeding-stuff with excreta.

Collection of semen and blood. The procedure for the collection of boar semen was that of Wallace (1949); the artificial vagina was a modification of that used by Ito, Niwa & Kudo (1948), as described by Glover & Mann (1954). The volume of the semen varied from 350 to 560 ml./collection. After removal of the seminal gel by filtering through a Büchner funnel (without paper), the filtrate was centrifuged at 1000 *g* to separate the spermatozoa from the seminal plasma. The spermatozoa were washed twice with 5 vol. of Ringer solution, and the washing fluids added to the seminal plasma. Blood was collected from the auricular vein, in the presence of heparin.

Fractionation of seminal plasma and sperm. The sperm and seminal plasma separated by the method outlined above were further fractionated.

The spermatozoa, after washing with Ringer solution, were suspended by grinding in ethanol (5 vol.), centrifuged after 1 hr., re-extracted with ethanol (5 vol.), then with ethanol–ether (3:1) and finally with ether. In this manner two fractions were obtained from the sperm, the ethanol–ether-soluble portion, which represents chiefly sperm lipids, and the ethanol–ether-insoluble portion, which consists mainly of deoxyribonucleoprotein and other intracellular sperm proteins (cf. Mann, 1954). Of these two fractions, the first was concentrated *in vacuo* to one-twentieth of the original volume, whereas the ethanol–ether-insoluble protein fraction was dried, yielding 1–3 g. of powder/ejaculate.

The seminal plasma was treated with ethanol (4 vol.), centrifuged after 1 hr. and thus separated into the ethanol-soluble portion, which contains ergothioneine (Mann & Leone, 1953), and the ethanol-insoluble protein fraction. The ethanol extract from the seminal plasma was concentrated *in vacuo* at pH 6 to one-tenth of the original volume of semen, whereas the ethanol-insoluble protein fraction was washed first with ethanol and then with ether, yielding 5–15 g. of powder/ejaculate.

Fractionation of sulphur compounds in seminal plasma. The method of Heath *et al.* (1953) was used.

Administration of [^{35}S]*methionine.* DL-[^{35}S]Methionine (63 mg., 5·5 mc) was obtained from the Radiochemical Centre, Amersham. The radioactive material was dissolved in 300 ml. of water and administered to the boar by adding 100 ml. of the solution to the daily food ration, on three consecutive days. Care was taken to ensure that all food was eaten by the animal.

Radioactivity measurements. Samples were counted in 1 cm.^{2} planchets, with a thin mica end-window Geiger tube. Samples which gave low or zero activities were subsequently measured with a windowless gas-flow counter, constructed as described by Garrow & Piper (1955).

Ergothioneine estimations. These were made by the method of Hunter (1949).

Ion-exchange resin. Zeo-Karb 225, 4½% cross-linked, 100–150 mesh, was obtained from the Permutit Co., London. Ergothioneine can be quantitatively eluted from this resin with 0·1 N-NH_3 soln. in a small sharp band; with higher degrees of cross-linkage, ergothioneine is not quantitatively eluted and the elution band is broad. With the 4½% cross-linked resin, ergothioneine is displaced by 0·1 N-NH_3 soln. immediately before the acidic amino acids and not, as one would expect, with the basic amino acids.

Ethanol purification. The ethanol used for the alumina chromatography was purified by distillation from magnesium and iodine. The distillate was redistilled from zinc.

Isolation of ergothioneine from seminal plasma

Extraction with ethanol. Seminal plasma (390 ml.), obtained from semen (480 ml.) as described above, was deproteinized with ethanol (1·6 l.), and the filtrate, adjusted to pH 6 with acetic acid, was concentrated under reduced

pressure to 40 ml. Ethanol (200 ml.) was added and, after standing at −10° for 2 hr. and centrifuging, the clear, pale-yellow supernatant was concentrated under reduced pressure almost to dryness. The residue was dissolved in water and made up to 250 ml. Suitable portions of this solution were used for ergothioneine estimation.

Ion-exchange chromatography. The method of Westall (1952) yielded an ergothioneine-rich fraction suitable for secondary purification by alumina chromatography (Melville, Horner & Lubschez, 1954). The solution described above was passed through a series of three columns (20 cm. × 1 cm.; 15 cm. × 1 cm.; 5 cm. × 0·8 cm.) of the Zeo-Karb 225, in the H^+ form. The columns were washed with water (100 ml.) and then with 0·1 N-NH_3 soln., the progress of displacement being followed by the change in colour of the resin. Fractions (5 ml.) were collected and one drop of each fraction was tested on filter paper for the presence of ergothioneine with 0·2% (w/v) ethanolic 2:6-dichloroquinonechloroimide (British Drug Houses Ltd.). A brick-red colour, which appears as soon as the ethanol evaporates, indicates the presence of ergothioneine; some aromatic compounds give a grey colour which appears more slowly. The fractions were also tested with ninhydrin. The fractions containing ergothioneine were combined and concentrated under reduced pressure nearly to dryness. The residue was transferred to a small glass dish and drying was completed over H_2SO_4.

Alumina chromatography. Activated alumina (type 'H', Peter Spence and Sons, Widnes), 100–150 mesh, was washed four times by decantation with a mixture of purified ethanol (300 ml.), water (100 ml.) and formic acid (4 ml.). A column (30 cm. × 1 cm.) was prepared from the resulting slurry. The total residue containing ergothioneine was transferred to the column with the minimum amount of the aqueous ethanol–formic acid mixture, and the column was then developed with the same solvent, 2 ml. fractions being collected. Each fraction was tested, as described above, for the presence of ergothioneine and amino acids. Ergothioneine was eluted in the fractions representing 20–50 ml. of solvent flow, whereas the amino acids were not eluted before 180 ml. of solvent had been passed. All fractions containing ergothioneine were combined and evaporated to dryness. In order to convert the ergothioneine into the free base and remove traces of aluminium compounds, the residue was dissolved in water and chromatographed on a 5 cm. × 0·8 cm. column of the Zeo-Karb 225 H^+ form. After washing with water, 0·1 N-NH_3 soln. was applied and the fractions containing ergothioneine were collected, combined and evaporated to dryness.

The dried residue was exhaustively extracted with cold dry ethanol and the crystals of insoluble ergothioneine were filtered off and washed with ethanol and ether. The ergothioneine (65 mg.) thus obtained was dissolved in water (2 ml.) and filtered. The filtrate and washings were concentrated to 2 ml. and tartaric acid (40 mg.) was added to the hot solution. On cooling, crystallization rapidly occurred. The resulting ergothioneine tartrate was recrystallized from aqueous ethanol (wt. 74 mg.).

Criteria of purity of ergothioneine tartrate. Melting point 235°; mixed m.p. with ergothioneine tartrate, 235°. (Melting points were determined on a Gallenkamp electrically heated micro-melting-point apparatus, and were uncorrected.) When analysed by the method of Hunter (1949) the isolated ergothioneine tartrate was shown to be 100% pure. By one-dimensional chromatography in butanol–acetic acid–water (60:15:25, by vol.) and treatment with dichloroquinonechloroimide, only one substance was revealed corresponding to ergothioneine (R_F 0·29); no ninhydrin-reacting substances were present.

Isolation of ergothioneine from blood

Red blood corpuscles separated by centrifuging from 350 ml. of heparinized blood were washed twice with 350 ml. of isotonic saline solution, lysed with an equal volume of water, and extracted with cold ethanol and $CHCl_3$ by the procedure of Mann & Keilin (1937). The haemoglobin-free extract thus obtained was concentrated *in vacuo* to remove ethanol. This solution (214 ml.) contained 7·7 mg. of ergothioneine (equivalent to 2·2 mg./100 ml. of whole blood). The procedure for the isolation of ergothioneine was the same as for the isolation from semen; wt. of recrystallized ergothioneine tartrate 6·8 mg.; m.p. 235°.

RESULTS

Experiments on the ethanol-soluble portion of seminal plasma

Details of the semen collections, volumes and the ergothioneine contents of seminal plasma are given in Table 1. Crystalline ergothioneine tartrate was isolated separately, by the procedure described above, from collections nos. I–V, and from a representative sample of the bulked collections nos. VI–XI. Each sample was rigorously purified and tested quantitatively by the diazo method of Hunter and by melting point and paper chromatography to establish purity and identity of the product. The samples were then 'counted' either as ergothioneine tartrate or after oxidation with bromine–water, as barium sulphate, at infinite thickness in 1 cm.^{2} planchets, both with the end-window Geiger tube and the windowless gas-flow counter. In no case was any detectable radioactivity above background observed, even on prolonged counting.

Portions of the ethanol extracts of seminal plasma, collections nos. II–V, were combined and then separated into two fractions representing the free plus ethereal sulphates and the bromine-oxidizable sulphur respectively. The barium sulphate samples thus isolated were 'counted' at a thickness of 2 mg./cm.^{2} rather than at infinite thickness, owing to the small amount of material obtained from the free plus ethereal sulphate fraction. The total activities were 141 counts/min. for the free plus ethereal sulphate fraction and 420 counts/min. for the bromine-oxidizable sulphur fraction. The amount of barium sulphate isolated from the bromine oxidation was equivalent to 95% of the ergothioneine present, as determined by the Hunter diazo test. The method of isolation was almost quantitative and as the ergothioneine after isolation proved to be inactive (see above), the

Table 1. *Data on collections of boar semen and the ergothioneine content of seminal plasma*

Before the administration of radioactive methionine, three collections were made, *A*, *B* and *C*, and of these *A* was analysed for ergothioneine. The radioactive compound, 5·5 mc of DL-[^{35}S]methionine (63 mg.), was dissolved in 300 ml. of water and 100 ml. fed to the animal on three consecutive days, beginning 31 May 1955.

No.	Date	Time after last feeding of ^{35}S (days)	Vol. of seminal plasma (ml.)	Ergothioneine (mg./100 ml. of seminal plasma)	Total ergothioneine (mg.)
A	17. v. 55	—	270	33·3	90·0
B	26. v. 55	—	—	—	—
C	31. v. 55	—	300	—	—
I	6. vi. 55	4	300	24·4	73·1
II	10. vi. 55	8	350	12·7	44·6
III	13. vi. 55	11	310	13·5	42·0
IV	18. vi. 55	16	360	11·1	40·0
V	21. vi. 55	19	360	12·5	45·0
VI	27. vi. 55	25	235	9·5	22·4
VII	4. vii. 55	32	350	9·1	32·0
VIII	11. vii. 55	39	305	7·8	23·8
IX	18. vii. 55	46	325	7·6	24·6
X	8. viii. 55	67	340	8·1	27·6
XI	24. viii. 55	83	370	7·0	25·5

radioactivity of the bromine-oxidizable sulphur fraction must reside in a small quantity of a relatively highly labelled substance.

Experiments on the ethanol-insoluble fraction of seminal plasma

As can be seen from Table 1, the semen collections were made from 4 to 83 days after the administration of 5·5 mc of [^{35}S]methionine, and in order to determine the general level of labelling of the body sulphur, separate samples of the ethanol-precipitated protein from the seminal plasma of collections nos. I and XI were oxidized (Heath *et al.* 1953) and the sulphur was isolated as barium sulphate. At the times of collection the respective counts of seminal plasma protein I and XI were 2608 and 325 counts/min. at infinite thickness and 1 cm.^{2} area by the end-window tube. It is apparent that the general level of labelling of the sulphur in the seminal plasma protein was sufficiently high during the whole course of the experiment for radioactivity to have been detected in the isolated ergothioneine if biosynthesis from cystine or methionine had taken place.

Experiments with blood

Ergothioneine was isolated from a sample of blood withdrawn 49 days after the administration of the radioactive methionine. This was counted as ergothioneine tartrate (6 mg./cm.^{2}) but no activity above background was observed. A portion of this blood plasma was oxidized and the sulphur isolated as barium sulphate; it had an activity of 538 counts/min./cm.^{2} at infinite thickness when counted with the end-window tube.

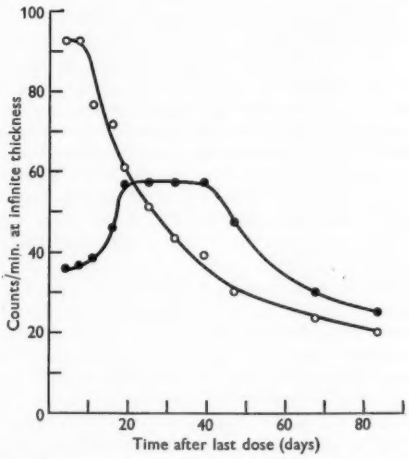

Fig. 1. Radioactivity of $BaSO_4$ prepared by oxidation of the total sulphur in boar seminal-plasma protein (○) and sperm protein (●) obtained after feeding 5·5 mc of DL-[^{35}S]methionine.

Experiments to determine the time required for the appearance of radioactive sulphur in the proteins of ejaculated spermatozoa and seminal plasma

As shown previously, after feeding [^{35}S]methionine to a boar, the isotope appears in the proteins of the seminal plasma much sooner than in the ejaculated spermatozoa (Heath *et al.* 1953). This observation was extended by further studies, the results of which are illustrated in Fig. 1. At various time intervals, ranging from 4 to 83 days after the

administration of [^{35}S]methionine, the proteins were separated from the sperm and seminal plasma of ten collections, and radioactivity measurements carried out by counting at infinite thickness after the main experiment was completed. All counts were made on the same day so that decay corrections were not necessary. As can be seen from Fig. 1, the seminal-plasma proteins of ejaculated boar semen show a high degree of labelling within a few days of feeding [^{35}S]methionine, and soon afterwards the radioactivity declines sharply. In contrast to the seminal plasma, the spermatozoa show only a low activity during the first 2 weeks after the administration of radioactive sulphur and attain a high level much later, namely, during the period between 20 and 40 days after feeding.

DISCUSSION

By an improved technique, which is generally applicable to other complex mixtures, pure crystalline ergothioneine was isolated from eleven collections of semen, taken from 4 to 83 days after the administration of 5·5 mc of DL-[^{35}S]methionine to a 7-year-old boar weighing 300 kg. In no case was any detectable radioactivity observed in the isolated ergothioneine. Ergothioneine was also isolated from the blood 49 days after the feeding of the isotope, and again the ergothioneine was not demonstrably radioactive. On the other hand, the general labelling of the sulphur of both the seminal plasma and the blood plasma at all times when samples of semen were taken for ergothioneine isolation was high enough, even for a 300 kg. experimental animal, for radioactivity to have been detected in the isolated ergothioneine if either direct biosynthesis or even a dynamic interchange of sulphur between ingested ergothioneine and other radioactive sulphur compounds had taken place. When viewed with respect to the dynamic state of other body constituents such as glutathione, which in the erythrocytes of normal man has a half-life of approximately 4 days (Dimant, Landsberg & London, 1955), the biochemical inertness of ergothioneine in such vital body fluids as blood and semen is remarkable. Dietary ergothioneine is known to be incorporated slowly into blood, where it occurs intracellularly (Heath, Rimington, Searle & Lawson, 1952), and rapidly into the seminal plasma, which is an extracellular fluid (Heath *et al.* 1953). As no ergothioneine is detectable in blood plasma the possibility could be envisaged that one source of seminal ergothioneine might be ergothioneine released from the erythrocytes as a result of their breakdown. Bush *et al.* (1955) have shown that the mean red-cell survival time in pigs is 62 days. In the present study no radioactivity was found in the ergothioneine isolated from the blood 49 days after the administration of radioactive isotope and from semen up to 83 days. Therefore the possibility that the site of biosynthesis of ergothioneine is connected with the erythropoietic system and that seminal ergothioneine is at least in part derived from the breakdown of matured erythrocytes appears improbable.

Under the conditions of the present experiment biosynthesis of ergothioneine did not occur. This experiment differed from our previous experiment (Heath *et al.* 1953) in that certain cereals such as oats and maize were rigorously excluded from the diet. It has been established (Baldridge, 1955; Melville *et al.* 1955) that these foodstuffs do increase blood ergothioneine levels. Recently Melville & Eich (1956) isolated ergothioneine from a sample of commercial rolled oats. The whole history of the investigations of the source of ergothioneine has been full of contradictions. We have shown that synthetically prepared [^{35}S]ergothioneine, when fed to the boar, passes readily into the seminal plasma (Heath *et al.* 1953). Melville *et al.* (1955) have also shown that there are variations in the uptake of added dietary ergothioneine by rats and that zein adversely affects this uptake. Fraser (1951) has shown that there are marked variations in blood ergothioneine levels in persons of different race maintained on the same hospital diet for over 3 months; also rapid changes can occur after injury. Although the results presented here seem most adequately to confirm that biosynthesis of ergothioneine does not take place in the pig, we feel that any conclusion should be drawn with caution. Even if ergothioneine were not present itself in a diet, one or other dietary component might supply an essential precursor for the biosynthesis of ergothioneine in the body, and this contingency has to be reckoned with in interpreting experimental results.

The present investigation was conducted under better experimental conditions than our previous work (Heath *et al.* 1953); not only was the administered radioactivity more than five times greater but instead of relying upon sulphur fractionation, the ergothioneine was in all cases isolated as such and recrystallized to zero activity. The present results therefore confirm the work of Melville *et al.* (1955) and support the hypothesis that ergothioneine is not biosynthesized in the animal body. At the same time, our results point to the existence in boar semen of another compound containing sulphur which is oxidized to sulphate by bromine water. This is clearly established by the labelling of the bromine-oxidizable sulphur fraction after the administration of [^{35}S]methionine. It seems improbable that the unknown compound is either sulphide or sulphite, as the method of hydrolysis of ethereal sulphates involves boiling in acid solution

for 30 min. The use of bromine-oxidizable sulphur as a method of estimation (Touster, 1951) or fractionation (Heath *et al.* 1953) of ergothioneine can lead to erroneous conclusions. Unidentified sulphur compounds have recently been detected in blood by Smith & Tuller (1955).

The peculiar conditions which govern the occurrence and distribution of ergothioneine in semen and male accessory secretions certainly point to the existence of factors other than dietary. Ergothioneine, it should be noted, is secreted in the male reproductive tract only in certain well-defined glandular structures. Whereas in the boar, for instance, it is confined to the seminal vesicle and does not occur in the Cowper's gland, epididymis or elsewhere, in the stallion it originates in the ampullae (Leone, 1954; Mann, Leone & Polge, 1956). It is also worth noting that ergothioneine has been recently shown to occur in large concentrations in the male accessory secretions of two Insectivora, namely, the mole and the hedgehog (Mann, 1956). On the other hand, the semen of man, ram and bull appears to contain only negligible quantities of this base.

It has been shown that in ejaculated boar semen maximum radioactivity appears much earlier in the seminal plasma than in the spermatozoa. The delay with which radioactive sulphur appears in ejaculated spermatozoa is undoubtedly due to the fact that the processes of spermatogenesis, sperm maturation and sperm transport in the male reproductive tract require much more time than the secretory processes in the male accessory organs and the formation of seminal plasma.

SUMMARY

1. A new, generally applicable method of isolating pure ergothioneine from biological material is described.

2. DL-[^{35}S]Methionine (5·5 mc) was administered to a mature pig; no radioactivity could be detected in the blood ergothioneine after 49 days or in the seminal ergothioneine up to 83 days after the administration of radioactive isotope.

3. Under the feeding conditions of this experiment no biosynthesis or metabolic exchange of ergothioneine sulphur with other labelled body constituents took place.

4. An unidentified sulphur compound which is oxidizable to sulphate by bromine water is present in pig semen.

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Glycerylphosphorylcholine Diesterase Activity of Nervous Tissue

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A study of the demyelinating diseases of man reveals at once the need for more knowledge of the enzymic processes that may be specifically concerned with the metabolism and maintenance of the myelin sheath of the nerve fibres. Experimental nerve degeneration and an accompanying break-up of the myelin sheath can be produced in some species of animal, notably the hen, by the administration of certain organophosphorus anticholinesterase compounds, and we have already described the changes in the activity of the cholinesterases and of other enzyme systems in animals poisoned with these substances (Earl & Thompson, 1952; Earl, Thompson & Webster, 1953). Since, on present evidence, it is not possible directly to relate the nerve degeneration produced by these compounds to an inhibition of either true or pseudo-cholinesterase activity in the nervous system (Davison, 1953; Thompson, 1954), we decided to study more fully the other esterases and related enzymes that are present in this tissue.

Because of the complex nature of the phospholipids of nervous tissue, and the various types of esters contained in these compounds, it was decided to begin this study with an examination of the action of brain extracts on component parts of these lipids. The action of the phosphatases of brain on phosphorylcholine, phosphorylserine and phosphorylethanolamine, components of the lecithins, sphingomyelins and kephalins, has already been reported (Strickland, Thompson & Webster, 1956*a*, *b*).

Since, however, the phospholipids are diesters of phosphoric acid, the action of brain preparations on the water-soluble α-glycerylphosphorylcholine was next investigated. Little work appears to have been done on the enzymic hydrolysis of this compound, although Hayaishi & Kornberg (1954) reported the presence of an adaptive glycerylphosphorylcholine diesterase in a strain of *Serratia plymuthicum*. The present study has, for reasons given above, centred round the nervous system, and a preliminary statement of some of our early results has already appeared (Thompson, 1956). While this work was in progress, Dawson (1956) described the presence of a glycerylphosphorylcholine diesterase in various rat tissues; certain differences, however, appear to exist between the enzyme studied by him in rat liver and our own findings in rat brain.

EXPERIMENTAL

Enzyme preparations. Aqueous homogenates (1 g. of tissue in 3 ml. of water) of the various tissues were prepared with a modified Potter–Elvehjem type of homogenizer. These homogenates were used either fresh or after dialysis in sausage-skin sacs for 20–24 hr. at 0–4° against approx. 100 vol. of water.

The following tissues were studied. Human: cerebral cortical grey matter, subcortical white matter, caudate nucleus, cerebellum, lower thoracic and lumbar spinal cord, and sciatic nerve. Dog and rabbit: grey and white matter (cerebrum). Hen: whole fore-brain, cerebellum, spinal cord and sciatic nerve. Rat and guinea pig: whole fore-brain, cerebellum and spinal cord. In the experiments with hen sciatic nerve, weighed amounts of the fresh minced tissue were used; human sciatic nerve was homogenized in a Waring Blendor and strained through muslin.

Buffer solutions. All the buffer solutions were adjusted to the required pH, a Cambridge pH meter being used. Acetate buffer was used for the range pH 4·5–5·5, phosphate for pH 6·5–7·5, and carbonate–bicarbonate for pH 8·5–10·5. The concentrations of the buffers are stated in the Results section.

For the manometric experiments, 0·025 M-$NaHCO_3$, gassed with $N_2 + CO_2$ (95:5, v/v), was used.

Substrate. A specimen of crystalline α-glycerylphosphorylcholine (GPC), prepared from ovolecithin, was kindly provided by Professor C. S. McArthur. A small amount of the crystalline $CdCl_2$ complex of GPC was also prepared in this laboratory according to the method of Tattrie & McArthur (1955), and was used, after removal of the cadmium on an ion-exchange resin (Zeo-Karb 225, Na form), in some of the later experiments described in this paper. The rate of enzymic hydrolysis of this latter preparation was very slightly less than that obtained with the crystalline free ester, under comparable conditions; this difference was shown not to be due to traces of cadmium remaining in solution, but may have been associated with the increased ionic strength of the reaction mixture owing to the presence of NaCl in the substrate solution as a result of the ion-exchange treatment (see Results).

Enzyme assay. For each experiment stoppered tubes were set up containing 1 ml. of brain homogenate (≡ 250 mg. of fresh brain), 1·5 ml. of buffer and 0·3 ml. of water (or of a solution of activator or inhibitor). The tubes were placed in a water bath at 38°, and after 10 min. 0·2 ml. of the substrate solution was added to each tube, to give a final concn. of 0·02 M. Samples (1 ml.) were then immediately removed and transferred into tubes containing 1·5 ml. of 8·3% (w/v) trichloroacetic acid. After 60 min. at 38° further samples were treated in the same way. After filtering off the precipitated proteins and phospholipids, 0·2 ml. portions of

each filtrate were taken for estimation of free choline, and 1 ml. samples for estimation of inorganic phosphate.

Estimation of choline. Free choline was estimated by the method of Appleton, La Du, Levy, Steele & Brodie (1953), modified by the use of $CHCl_3$ instead of ethylene dichloride as the solvent for the choline periodide (Webster, 1956).

Estimation of inorganic phosphate. Inorganic phosphate was estimated by the method of Ernster, Zetterström & Lindberg (1950), according to the procedure outlined by Strickland *et al.* (1956*a*).

RESULTS

Evidence for hydrolysis of GPC by brain

Initial experiments, to determine whether GPC is actively hydrolysed by brain, were carried out by estimating free choline and inorganic phosphate in the reaction mixture, after incubating GPC with homogenates of dialysed rat brain. In order to follow acid production, these experiments were carried out at pH 7·4 in 0·025 M-$NaHCO_3$ buffer, and the CO_2 evolution was simultaneously measured by the Warburg technique. The results are summarized in Table 1.

It will be seen that at pH 7·4 GPC is rapidly hydrolysed by dialysed homogenates of rat brain. Further, it will be noted that approximately 6 moles of choline are liberated for each mole of inorganic phosphate set free. This would suggest that choline is first split off from the substrate, with a subsequent smaller release of inorganic phosphate from α-glycerophosphate, rather than that phosphorylcholine is first formed, secondary hydrolysis of which would, of course, release free choline and inorganic phosphate in equimolar proportions. It must be remembered also that it has been shown by Strickland *et al.* (1956*a*, *b*) that at pH 7·4 both α-glycerophosphate and phosphorylcholine are attacked only slowly by the phosphomonoesterases of brain. It should also be pointed out that under the conditions used here phosphorylcholine is not estimated by the periodide method (Appleton *et al.* 1953; Webster, 1956).

Table 1. *Free choline, inorganic phosphate and acid production from GPC by dialysed rat-brain homogenates*

pH 7·4, in 0·025 M bicarbonate buffer, and in the presence of mM-Mg^{2+} ions.

Expt. no.	μmoles liberated/g. of moist tissue/hr. CO_2	Choline	Inorganic phosphate	Molar quotient Choline/phosphate
1	9·6	27·2	4·1	6·6
2	17·1	27·0	4·2	6·4
3	8·0	22·1	4·3	5·2
4	25·1	35·8	6·0	6·0
Average	15·0	28·0	4·6	6·0

Although the amount of choline liberated showed some variation in different experiments, it will be seen (Table 1) that the CO_2 evolution varied much more widely, ranging from 8·0 to 25·1 μmoles/g./hr. This variability of measured CO_2 output can probably be attributed to the high pK of the α-glycerophosphoric acid formed, and will be discussed more fully below.

It was found that dialysis had a negligible effect on the activity of these brain homogenates, although subsequent acetone-drying of the dialysed preparations resulted in about 36 % loss of activity, even in the presence of mM-Mg^{2+} ions.

Effect of pH

The pH–activity curve for the hydrolysis of GPC by rat-brain homogenates is shown in Fig. 1. In these experiments the final buffer concentration was 0·06 M, acetate being used for the range pH 4·5–5·5, phosphate for pH 6·5–7·5, and carbonate–bicarbonate for pH 8·5–10·5. It will be seen that under our conditions the enzyme shows maximal activity at about pH 9·5, although also showing considerable activity at the physiological pH.

It will be noted, however, that the rate of hydrolysis at pH 7·5 in 0·06 M phosphate buffer (Fig. 1) is approximately 40 % lower than the rate at pH 7·4 in 0·025 M-$NaHCO_3$ buffer (Table 1), and it seemed possible that variations in the ionic strength of the incubation medium might affect the apparent activity of the enzyme. Experiments were therefore carried out to test this point. Thus addition of NaCl to incubation media, initially 0·025 M with respect to carbonate–bicarbonate buffer, to give a final total $[Na^+]$ and $[Cl^-]$ equivalent to 0·05 and 0·025 M respectively, at pH 8·9 caused 19 % inhibition of activity, whereas twice this amount of added NaCl caused a 27 % fall. Further, increasing the strength of the carbonate–bicarbonate buffer from 0·025 to 0·1 M caused a 27 % decrease in apparent activity.

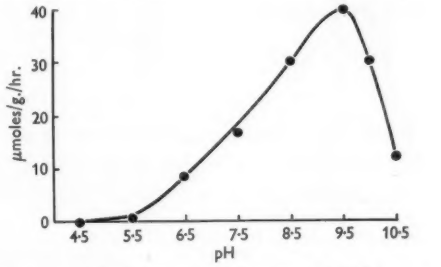

Fig. 1. pH–Activity curve for hydrolysis of GPC by rat-brain homogenates. Buffers: pH 4·5–5·5, 0·06 M acetate; pH 6·5–7·5, 0·06 M phosphate; pH 8·5–10·5, 0·06 M carbonate–bicarbonate. (Results expressed as μmoles of choline/g. of fresh tissue/hr.)

Activation by metals

In view of the known activation of many of the phosphomonoesterases by Mg^{2+} ions, the sensitivity of this glycerylphosphorylcholine diesterase to metal ions was studied.

Rat-brain homogenates, dialysed for 24 hr., showed no significant activation by mM-Mg^{2+} ions either at pH 7·4 or at pH 8·9. Equivalent concentrations of Mn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} ions also failed to show any significant activating or inhibitory effects. Since it was possible that this period of dialysis might have been insufficient to remove relatively firmly bound metals, it was decided to study next the effect of addition of ethylenediaminetetraacetic acid (EDTA). It was found that mM EDTA caused 100% inhibition both of free choline release and of liberation of inorganic phosphate.

In view of this abolition of activity by EDTA, the possible activating effect of various metal ions on the EDTA-treated enzyme was next examined. For this purpose a brain homogenate was dialysed for 6 hr. at 0–4° with four changes of water (each of about 100 vol.). EDTA was then added to give a final concentration of 10^{-2}M, and dialysis was continued for a further 65 hr. at 0–4°, with two changes of water at 24 and 48 hr. respectively. The results of two such experiments are summarized in Table 2.

Table 2. *Effect of addition of various metal ions on enzymic hydrolysis of GPC by rat-brain preparations treated with EDTA and dialysed*

For details of treatment see text. Final concn. of added metal ions was mM. Results are expressed as μmoles of choline/g./hr. Numbers of experiments are given in parentheses.

	Activity	% of untreated value
Untreated (4)	36·4	—
No activator (2)	3·9	11
Mn^{2+} (2)	29·8	82
Mg^{2+} (2)	26·6	73
Ca^{2+} (2)	22·0	60
Co^{2+} (2)	16·6	46
Zn^{2+} (2)	9·6	26
Cu^{2+} (2)	6·2	17

The effect of EDTA treatment followed by dialysis was to reduce the activity from a mean level of 36·4 μmoles of choline/g./hr. to 3·9 μmoles of choline/g./hr. A very considerable degree of reactivation was brought about by the addition of either Mn^{2+} or Mg^{2+} ions; Ca^{2+} and Co^{2+} ions caused somewhat less reactivation, whereas with Zn^{2+} and Cu^{2+} ions the effect was small.

Effect of inhibitors

The complete inhibition of activity brought about by mM EDTA has already been mentioned.

Since the substrate used in this work is a choline ester it was of interest to discover whether recognized inhibitors of the cholinesterases had any effect on this diesterase; it was found that eserine (mM), di*iso*propyl phosphorofluoridate (mM) and tri-*o*-cresyl phosphate (18 mM) had no significant effect.

The enzyme was also fluoride-insensitive, and was not affected by the presence of mM cysteine hydrochloride.

Distribution in nervous tissue

Preliminary experiments were carried out with homogenates prepared from different regions of the nervous system, and also from different species, to discover whether any marked variations in the level of enzymic activity were present in different areas. The results of these experiments are shown in Table 3. It will be seen that the central nervous system of the rat is considerably more active than nerve tissue from any of the other species studied. Although the areas studied and the observations made are too few to warrant any firm conclusions, it would appear that, in general, the whole fore-brain and spinal cord show higher levels of activity than the cerebellum, cerebral grey and white matter, caudate nucleus or sciatic nerve.

DISCUSSION

The results described above indicate that GPC is rapidly hydrolysed by preparations of human, rat, guinea pig, dog and hen nervous tissue. With rat

Table 3. *Glycerylphosphorylcholine diesterase activity of different areas of nerve tissue from different species*

Expressed as μmoles of choline/g./hr. Measurements carried out with undialysed homogenates or, in the hen sciatic nerve, with minced tissue, at pH 8·9 in 0·025M-$NaHCO_3$ buffer. Numbers of determinations are given in parentheses.

	Whole fore-brain	Spinal cord	Cerebellum	Cerebrum		Caudate nucleus	Sciatic nerve
				Grey	White		
Rat	36·4 (4)	58·0 (2)	25·0 (2)	—	—	—	—
Guinea pig	19·8 (2)	12·1 (2)	12·0 (2)	—	—	—	—
Hen	13·4 (2)	9·5 (2)	2·8 (2)	—	—	—	1·6 (2)
Human	—	9·4 (2)	3·1 (3)	2·2 (5)	2·9 (4)	3·3 (3)	5·2 (3)
Rabbit	—	—	—	2·0 (2)	2·0 (2)	—	—
Dog	—	—	—	10·3 (2)	12·4 (2)	—	—

tissues it was found that dialysis of the brain homogenates had little effect on the final activity. Acetone-dried preparations of dialysed rat brain showed about 60 % of the original activity. It is of interest to point out that the fore-brain of the rat appears to be, under our conditions, more active than the preparations extracted from rat liver by Dawson (1956).

It has been stated by Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) that GPC is not hydrolysed by either the acid phosphatase of the prostate or the alkaline phosphatase of the intestine, and we have concluded from our observations that the liberation of choline from GPC by brain is also brought about by a diesterase distinct from the phosphomonoesterases which are active against phosphorylcholine, and which are known to be present in this tissue. Our reasons for this conclusion are: (i) approximately 6 moles of choline are liberated for each mole of inorganic phosphate set free; (ii) although the optimum pH for splitting is at about pH 9·5 there is considerable activity at pH 7·4, in contrast with the phosphomonoesterases of brain which show only negligible activity against phosphorylcholine at this pH; (iii) the presence of mM cysteine hydrochloride has no effect on the release of choline from glycerylphosphorylcholine, although causing approximately 60 % inhibition of hydrolysis of phenyl phosphate or of phosphorylethanolamine (Strickland *et al.* 1956*b*).

Although in the earlier experiments we attempted to follow the course of the hydrolysis of GPC by measuring liberation of carbon dioxide from a bicarbonate buffer at pH 7·4, we found large variations in the amounts of carbon dioxide released, relative to the amounts of choline and inorganic phosphate set free. This variability, we believe, is due to the fact that the splitting of choline from GPC will release a monosubstituted phosphoric acid which will be only partially dissociated at pH 7·4, so that only a fraction of the theoretical amount of carbon dioxide will be liberated, and this fraction will vary widely with any slight shift of the pH of the medium.

The influence of pH on the activity of this diesterase in brain, determined under our conditions, is markedly different from that noted by Dawson (1956) for the enzyme in rat liver. Whereas in the rat-liver preparations optimum activity was observed at about pH 7·5, the brain enzyme shows an optimum at about pH 9·5. This latter pH optimum and also the overall shape of the pH–activity curve of the brain enzyme are very similar to the findings of Hayaishi & Kornberg (1954) with the glycerylphosphorylcholine diesterase of *Serratia plymuthicum*.

The influence of substrate concentration on enzymic activity was also examined. The approximate Michaelis constant calculated from the slowly rising substrate concentration–activity curve appears to be about 2·7 mM. Hayaishi & Kornberg (1954) obtained a K_m of 2·5 mM for their bacterial diesterase. In agreement with these workers and also with Dawson (1956), the brain diesterase is completely inhibited by mM EDTA, although in contrast to the earlier findings with the bacterial and liver enzymes, we have not found the presence of zinc ions to be inhibitory to the brain enzyme.

SUMMARY

1. The hydrolysis of α-glycerylphosphorylcholine (GPC) by preparations of nervous tissue from various species has been studied.

2. GPC is rapidly hydrolysed to α-glycerophosphate and choline by a diesterase present in these tissues.

3. Although active at pH 7·4, maximal splitting of GPC was observed at pH 9·5.

4. The addition of mM ethylenediaminetetraacetic acid caused complete inhibition of activity, which could be largely restored by the addition of Mn^{2+}, Mg^{2+}, or Ca^{2+} ions.

5. The diesterase is not inhibited by fluoride, cysteine, eserine, di*iso*propyl phosphorofluoridate or tri-*o*-cresyl phosphate.

Our thanks are due to Professor C. S. McArthur for the gift of crystalline α-glycerylphosphorylcholine, and to the Medical Research Council for a grant to one of us (E. A. M.).

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Respiration Rate of Rat-Liver Cells at Low Oxygen Concentrations

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Liver damage following chronic oxygen lack has been demonstrated experimentally in rabbits by Martin, Bunting & Lowenhart (1916) and in dogs by Goldschmidt, Ravdin & Lucke (1937). A similar type of damage has been found in the livers of airmen dying of acute anoxia (Kritzler, 1944). Recently, Bassi & Bernelli-Zazzera (1955) have made a quantitative study of the changes occurring in the livers of rats made acutely hypoxic. They found that after the animals had been kept for 2 hr. in an atmosphere containing 3% of oxygen the liver cells became vacuolated and their metabolic pattern altered. It is reasonable to suppose that this change was brought about by an impairment of the oxygen supply to the liver cells. For this reason it would be interesting to determine the effect of low oxygen concentrations on the respiration rate of the liver, as has been done with many other biological systems.

The selection of the best biological preparation of liver to use raises some difficulties. Hill (1948) discussed this problem in relation to frog muscles, whose respiration rate he measured as a function of oxygen concentration. The slices which Cruickshank & Trotter (1956) found satisfactory in their studies of skin respiration would not be suitable, for histological reasons. The preparation must be such that the suspending medium containing oxygen bears a similar spatial relation to the cells as blood plasma does in the intact organ. Elias (1955) has shown that the cells of rat liver form a muralium, or sponge-like structure of plates, one cell thick. Thus, in general, the rat-liver cell receives its oxygen supply directly from the plasma without any other parenchymal cell intervening. Because of this it would be reasonable to study the respiration of individual cells suspended in an oxygenated solution. Such suspensions would have to be free of extraneous respiratory particles; since the removal of mitochondria from cell suspensions is very difficult because of their stickiness, the method of preparation should be so gentle that no cells are disrupted. Anderson (1953) has described a method of obtaining individual cells by softening the cement substance with calcium-complexing agents, and Dulbecco & Vogt (1954) have used trypsin for the same purpose. Since these methods introduce substances foreign to the cell, a method was developed which avoided this difficulty (Longmuir & ap Rees, 1956).

There are two general methods of measuring the respiration rate of a biological system as a function of oxygen concentration: (i) The manometric method used by Warburg & Kubowitz (1929) on bacteria and recently improved by Baender & Kiesse (1955) working with mitochondria; by this method, however, each experiment yields only one point on the curve of respiration rate against oxygen concentration and, because of the time required, could not be expected to give a complete curve on one preparation. (ii) The polarographic methods used by Winzler (1941) on yeast, Hill (1948) on frog muscle and by Longmuir (1954) on bacteria; these methods produce a complete curve in a few minutes and so are suitable for application to preparations which may not retain their activity for long. Since the results are produced as a continuous curve rather than as discrete measurements the latter methods more readily demonstrate such critical points as the concentrations of oxygen at which the respiration rate just begins to fall and at which it reaches its half value. For these reasons the polarographic method was used.

In order to obtain results comparable with those of Baender & Kiesse (1955), obtained with mitochondria, succinate was used as the substrate. The experiments of Baender & Kiesse (1955) were repeated with the polarographic technique.

MATERIALS AND METHODS

Solutions. The cells were suspended in a mixture of equal parts of 0·1M-KCl and 0·1M phosphate buffer, pH 7·3, prepared as previously described (Longmuir, 1954). To this was added 0·4M sodium succinate, the quantity of which was determined by preliminary experiments described below.

Cells. These suspensions were prepared by the methods of Anderson (1953) (slightly simplified), of Dulbecco & Vogt (1954) and of Longmuir & ap Rees (1956). The reagents used were as far as possible Polaritan (Hopkins and Williams) and the water was de-ionized. The activity of the preparations is expressed as q_{O_2} (N) (μl. of O_2/mg. of total N/hr.), because the determination of the wet or dry weight of cells suspended in a relatively large amount of saline is not very accurate.

In practice it was found that all methods gave similar results except that the preparation of Dulbecco & Vogt was rather more quickly inactivated by the experimental

technique used. After the preliminary experiments using all three methods the method of Longmuir & ap Rees (1956) was used exclusively, as it was found to be most convenient.

Mitochondria. These were prepared by the method of Klemperer (1955).

Methods. The method used is essentially that previously described (Longmuir, 1954) but with some improvements (Fig. 1). A self-centring ball race (3) replaces the journal bearing between the rod (5) and the disk (2) and the rod is connected to the electrode by insertion directly into the glass tube (6); the metal and glass are separated by a layer of rubber (4). Both the motor and the cell are securely attached to brass plates bolted to one another and also to the side of a thermostat bath (accurate to 0·01°). The connexion to the anode is made through a constricted continuation of the cell (10) below the sintered-glass disk (9). The agar is placed in this part instead of in the cell proper. A wide-bore polyvinyl chloride tube filled with saturated KCl makes connexion with the anode. The anode, which is sufficiently far away from the bath to avoid temperature effects, consists of a mercury pool (60 cm.²); this is superior to a calomel electrode (Heyrowsky, 1957). The mechanical improvements simplify the technique and make the diffusion current more stable. Provided that prepolarization and ageing are duly carried out this assembly gives current × voltage curves which fulfil Heyrowsky's (1957) criterion of reproducibility. In order to facilitate direct measurements of respiration rate a differentiating circuit is incorporated in the polarograph (Fig. 2). The reading of the galvanometer is a measure of the respiration rate of the biological system being studied.

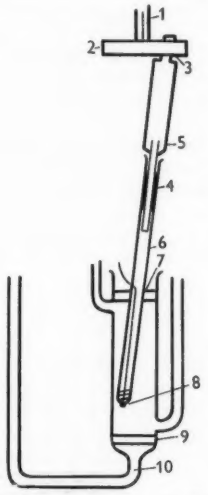

Fig. 1. Diagram of the apparatus. 1, Shaft of constant-speed motor; 2, disk; 3, self-centring bearing set into 2; 4, rubber tubing between eccentric shaft 5 and glass tube 6; 7, rubber disk; 8, gold electrode; 9, sintered-glass disk; 10, 2% agar in saturated KCl.

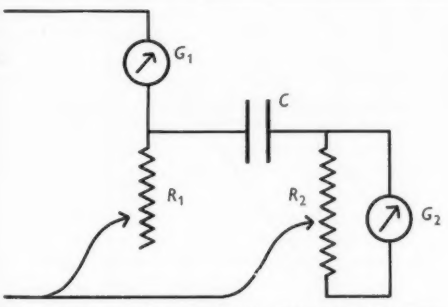

Fig. 2. Differentiating circuit. G_1, Recorder of the Cambridge Polarograph; G_2 galvanometer (125 scale divisions/μA); R_1, decade box 500–8000Ω; R_2, potential divider of 10 000Ω; C, paper condenser of 10 μF.

A different technique of re-aeration is used. Syringes (20 ml.) are attached to the inlet and outlet of the polarographic cell. These are each one-quarter full of a suspension of cells or mitochondria, and one-quarter full of air or oxygen. The polarographic cell contains only the suspension. When the oxygen in the suspension contained in the polarographic cell is exhausted, it can be renewed by drawing the liquid from one syringe to the other without introducing a gas phase into the cell.

RESULTS

Initial experiments showed that the activity of the liver-cell suspensions fell off while in the polarographic cell. The greatest rate of inactivation observed at 40° was 10%/hr. It was decided to perform all experiments quickly rather than devise a method of preventing inactivation.

The effect of succinate concentration on the q_{O_2} (N) of the liver-cell suspension was investigated. The reciprocal of the respiration rate was plotted against the reciprocal of the succinate concentration. The points lay on a straight line. The value of the maximum respiration rate and the concentration of succinate (Km) producing half the maximum rate were derived from this line by the method of Lineweaver & Burk (1934). The value of Km at 40° was found to be 1·03 mM. At lower temperatures it was lower. In all subsequent experiments a concentration of succinate (greater than 12 mM) sufficient to give the maximum respiration rate was used.

Fig. 3 shows the results of a typical experiment with an optimum concentration of succinate. Curve (*a*) is obtained from the recorder of the polarograph and is a plot of oxygen concentration against time. Curve (*b*) is a plot of respiration rate, the galvanometer reading, against time. In Fig. 4 the results are plotted in the more orthodox manner of respiration rate against oxygen concentration.

It will be seen that there is an appreciable scatter of the experimental points about the horizontal line in curve (*b*) of Fig. 3. It is not absolutely clear from this experiment alone that there is a discontinuity in

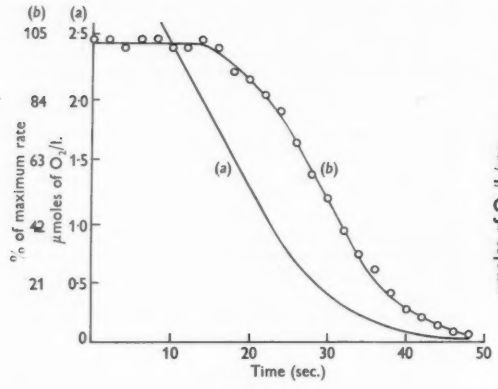

Fig. 3

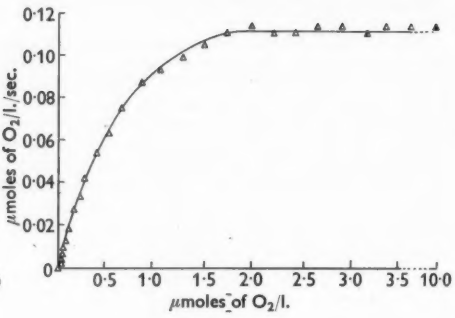

Fig. 4

Fig. 3. Plots of the oxygen concentration of the cell suspension and respiration rate of the liver cells against time. Curve (*a*) is the trace obtained from the recorder of the polarograph and its ordinate is expressed as μmoles of O_2/l. Curve (*b*) is obtained by plotting the readings of the galvanometer (the respiration rate) expressed as percentages of the maximum rate.

Fig. 4. Variation of the respiration rate of liver cells in μmoles of O_2/l./sec. (ordinate) with oxygen concentration in μmoles of O_2/l. (abscissa).

the plot of respiration rate against oxygen tension, the respiration rate beginning suddenly to fall at this value. However, it is possible to establish that this is so. The scatter is due to noise coming from an extraneous source and it is independent of the signal strength. Thus the error in this region can be reduced by increasing the amount of rat-liver cells placed in the polarographic cell. In experiments in which this was done it was found that the galvanometer reading remained constant and then fell abruptly, i.e. that the respiration rate does fall abruptly from the maximum. In these experiments the rate changes too rapidly to permit the accurate reading of points in the second part of the curve.

Fig. 5 shows plots of $\log v/(v_{max.}-v)$ against log (oxygen concentration) for experiments at two different temperatures, where v is respiration rate; the significance of this plot is discussed below. The results obtained with mitochondria gave similarly shaped curves.

Experiments were conducted at various temperatures between 1·40° and 40·00°. The logarithm of the respiration rate was plotted against the reciprocal of the absolute temperature, and gave a straight line. From the slope of this line the energy of activation of the whole system was found to be 16 000 cal. By a similar treatment, the logarithm of the concentration of oxygen at which the cytochrome oxidase is half saturated [calculated from the equation of Baender & Kiesse (1955)]

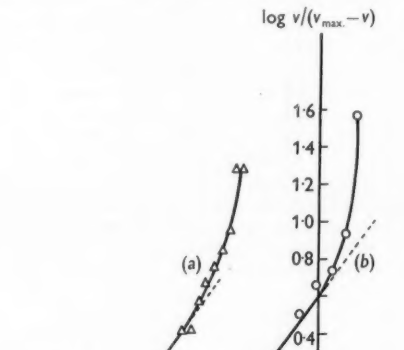

Fig. 5. A plot of $\log v/(v_{max.}-v)$ (ordinate) against log (oxygen concentration) (abscissa), showing the deviation from a straight line at higher respiration rates. Curve (*a*) at 10°, curve (*b*) at 40°.

being used, the value of the heat of the reaction was found to be 13 500 cal.

In all the experiments in which the respiration rate was varied by altering either the concentration of succinate or the temperature it was found that the concentration of oxygen (K) at which the cells respired at one-half of their maximum rate was linearly related to the maximum respiration rate within the limits of experimental error (average coefficient of variation 4 %). The relationship obtained by least squares is $K = \alpha q_{O_2}(N) + \beta$, where $\alpha = 1{\cdot}2 \times 10^{-9}$ and $\beta = 4{\cdot}26 \times 10^{-8}$.

DISCUSSION

It has been held for some time (Tang, 1933) that the relationship between the respiration rate (v) of various biological systems and the partial pressure of oxygen (p_{O_2}) is

$$v = \frac{p_{O_2}}{K_1 + K_2 \times p_{O_2}}, \qquad (1)$$

where K_1 and K_2 are constants. Baender & Kiesse (1955) have recently shown that for mitochondria, for a bacterium and for a yeast, the relationship is

$$v = \frac{v_{max.} \times p_{O_2}^{1{\cdot}4}}{K + p_{O_2}^{1{\cdot}4}}, \qquad (2)$$

where $v_{max.}$ is the maximum respiration rate. They obtained this by plotting $\log v/(v_{max.} - v)$ against $\log p_{O_2}$, when they found that between 10 and 90 % of the maximum rate, their full experimental range, the points lay on a straight line of slope 1·4. In Fig. 5 the results obtained with liver cells at two different temperatures are plotted in this way. In the straight region the slope is 1·4 but the upper ends rise more steeply. This means that the respiration rate approaches the maximum at a lower oxygen concentration than predicted by either of the equations. Baender & Kiesse (1955) reported that at three times the concentration of oxygen at which the respiration rate was half maximal the respiration rate had just begun to fall, and called this the 'critical oxygen concentration'; but later in their paper they assume that their equation is valid at higher concentrations, i.e. that the respiration rate approaches the maximum rate asymptotically. There is thus no theoretical 'critical oxygen concentration'.

The curves shown in Figs. 3 and 4 show that with liver cells there is a true 'critical oxygen concentration' which is between four and five times the value of the oxygen concentration at which the rate is one-half of the maximum. According to Eqns. (1) or (2) the respiration rate at this critical value should be only 80 or 90 % of the maximum respectively. The instrumental error in the determination of the respiration rate is about 3 %, and it is less in experiments designed to clarify this region.

There are two possible explanations for this limitation of the respiration rate. A reduction of the concentration of oxygen in the suspending medium eventually results in anoxia of those mitochondria furthest from the surface of the cell. This concept has been treated mathematically by Gerard (1931) and Rashewsky (1933) for a somewhat simplified system; the respiration rate, however, seems to fall off rather more sharply than these equations require. An alternative explanation is that above 80 % saturation of the respiratory enzyme another component of the succinic oxidase system becomes limiting. Winzler (1941) found this to be true in yeast. He changed the limiting dehydrogenase by altering the substrate and found indirectly that the percentage saturation of cytochrome oxidase at the maximum respiration rate was changed. By direct spectroscopic examination Chance (1952) has shown that in the steady state, when the respiration rate is maximal, the cytochrome a_3 of heart-muscle preparation is only 87 % oxidized. If this explanation is correct, the respiration rate of mitochondria above 80 % of the maximum should rise with increasing oxygen concentration more rapidly than predicted by Baender & Kiesse (1955). Experimentally, with the technique described in this paper, it was found that the respiration rate of mitochondria did increase in this way. These results do not contradict those of Baender & Kiesse (1955); the deviation from the straight line occurs in a region of respiration rate which they did not investigate fully.

The values obtained for the energy of activation and the heat of reaction are the same, within the limits of experimental error, as those found by Baender & Kiesse for mitochondria. This suggests that fundamentally the same process is being studied in both cells and mitochondria and that diffusion through the cell cytoplasm is not a limiting factor, as it may be in large bacteria (Longmuir, 1954). Direct comparison of the oxygen concentrations at which mitochondria and cells respire at one-half of their maximum rate is difficult owing to the marked variation of these oxygen concentrations with q_{O_2}, but a comparison with the figures of Baender & Kiesse suggests that the value for cells with a comparable respiration rate is slightly higher.

SUMMARY

1. A method of determining simultaneously the respiration rate of a biological system and the concentration of oxygen in the suspending medium is described.

2. This method has been used to measure the relation between the respiration rate of rat-liver cells and oxygen concentration.

3. The significance of the relationship is discussed.

I am indebted to Professor A. Haddow of the Chester Beatty Research Institute for the supply of rat livers and to Mrs Wendy ap Rees for technical assistance.

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The Structure of the Naturally Occurring Phosphoglycerides

3. ACTION OF MOCCASIN-VENOM PHOSPHOLIPASE *A* ON OVOLECITHIN AND RELATED SUBSTANCES*

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The enzyme phospholipase *A*, which is present in the venom of several species of snake, removes a single fatty acid radical from the naturally occurring lecithins to give the corresponding lysolecithins. Lecithin, when isolated from biological materials by solvent-fractionation procedures, usually contains saturated and unsaturated fatty acid radicals in approximately equimolar proportions; on treatment with phospholipase *A*, the unsaturated fatty acid radicals are found to be preferentially removed (Levene & Rolf, 1923; Chargaff & Cohen, 1939; Fairbairn, 1945). However, the enzyme does not act specifically on unsaturated fatty acid ester linkages, since Zeller (1952) and Hanahan, Rodbell & Turner (1954) have demonstrated the removal by phospholipase *A* of a single fatty acid radical from synthetic dimyristoyl-L-α-lecithin and yeast dipalmitoleyl-lecithin respectively. In the first case, the lecithin contains two saturated fatty acid radicals; in the second, both are unsaturated. It has been shown by Hanahan (1954) and by ourselves (Long & Penny, 1954) that it is the fatty acid radical attached to the α′ position in the lecithin molecule which is selectively detached by phospholipase *A*.

In the present work, the phospholipase *A* of cottonmouth-moccasin venom has been studied with regard to its action on ovolecithin and other naturally occurring and synthetic phosphoglycerides. The enzyme has been found to require Ca^{2+} ions and to be specific for the L-α form of synthetic lecithins; phosphatidylethanolamine and probably certain other naturally occurring phosphoglycerides have also been found to act as substrates. Some of this work has been published in preliminary form (Davidson, Long & Penny, 1955).

* Part 2: Long & Maguire (1954).

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EXPERIMENTAL

Materials

Source of phospholipase A. Samples of the dried venom of the cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*), rattlesnake (*Crotalus adamanteus*) and cobra (*Naia naia*) were purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. Aqueous solutions (0·1%) were used either immediately or after storage for up to 24 hr. at 2°. Six samples of moccasin venom were examined; all showed a significantly higher phospholipase *A* activity than did the rattlesnake or cobra venom, and were therefore used in the experiments to be described.

Ovolecithin. The ovolecithin used in most experiments was prepared by the method of Pangborn (1951), which involves purification through the cadmium complex. The average I_2 val. was 54 and the ratio equivalents of ester:atoms of P (ester:P ratio) was about 1·89:1; such preparations contain about 10–15% of lysolecithin (Lea & Rhodes, 1954). Samples of ovolecithin free from lysolecithin were obtained by silica-gel chromatography according to Lea, Rhodes & Stoll (1955).

Egg phosphatidylethanolamine. A pure specimen was given to us by Dr C. H. Lea. Other samples were prepared according to Lea *et al.* (1955).

Ox-brain 'kephalin' fractions. (*a*) One of the samples used was prepared by Long & Maguire (1953) and corresponded with 'fractions III–V' of Folch (1942), i.e. it consisted largely of a mixture of phosphatidylethanolamine and phosphatidylserine, contaminated with plasmalogens. (*b*) Another sample was prepared from the acetone-insoluble ethanol-soluble fraction of ox-brain lipids, obtained by the procedure of Folch (1942). After removing the ethanol by evaporation under reduced pressure, only part of the residue dissolved in absolute ethanol. The ethanol-insoluble material was dissolved in $CHCl_3$–methanol (4:1, v/v) and chromatographed on silica gel according to Lea *et al.* (1955). The material eluted from the column was free from lecithin and occupied the position expected for phosphatidylethanolamine. However, it was heavily contaminated with plasmalogen and had an ester:P ratio of 1·58:1.

Other naturally occurring phosphoglycerides. The following preparations were given to us: 'phosphatidylinositol' (Hawthorne, 1955) from liver by Dr J. N. Hawthorne, diphosphoinositide (Folch, 1949) from brain by Dr J. Folch, and dipalmitoyl-lecithin from yeast (Hanahan *et al.* 1954) by Dr D. J. Hanahan. The latter had an ester:P ratio of 1·84:1.

Synthetic phosphoglycerides. The following compounds were given to us by Drs T. Malkin and T. H. Bevan: dipalmitoyl-DL-α-lecithin, distearoyl-DL-α-lecithin and dipalmitoyl-β-lecithin. Synthetic dimyristoyl-L-α-lecithin was a gift from Dr E. Baer.

Ether. The ether (British Drug Houses, AnalaR) was washed with a solution of $FeSO_4$, dried over $CaCl_2$ and distilled. It was free from peroxides and from esters (as determined by the hydroxamic acid method).

Analytical methods

Total phosphorus was converted into inorganic P by ashing an amount of phospholipid, containing not more than 0·2 μg. atom of P, with 0·3 ml. of 60% $HClO_4$; the inorganic P was determined by the method of Berenblum & Chain (1938), as modified by Long (1943). Ester content was measured by the hydroxamic acid method described by Shapiro (1953); a pure sample of ethyl palmitate, given by British Industrial Solvents (Distillers Co. Ltd.), was used as a standard.

All colorimetric determinations were made with a photoelectric colorimeter (Evans Electroselenium Ltd.).

RESULTS

Action of moccasin-venom phospholipase A on ovolecithin

All the experiments described in this section were carried out on samples of ovolecithin prepared by the procedure of Pangborn (1951). When the method of Hanahan *et al.* (1954) was used to follow the action of the various snake venoms on an ethereal solution of ovolecithin, consistent results were obtained only when Ca^{2+} ions were added to the reaction mixture. The following modification proved satisfactory. Into each of several matched test tubes (125 mm. × 15 mm.) was pipetted 1 ml. of dry peroxide-free ether containing 1·5–2·0 μmoles of ovolecithin. At fixed time intervals, 0·01 ml. of an aqueous venom solution (1 mg. of venom/ml. of 0·005 M-$CaCl_2$) was added to each tube. The contents were mixed and the tubes allowed to stand at room temperature (usually 18°). Lysolecithin, which is insoluble in ether, was slowly precipitated as the reaction proceeded. After 5–120 min., enzymic activity was stopped by adding 2 ml. of ester-free ethanol, which also dissolved the lysolecithin. Ester content was determined by the hydroxamic acid method, the red–brown colours being measured against a standard ethyl palmitate solution in ether–ethanol and an ester-free ether–ethanol blank. In the zero-time sample containing ovolecithin, the ethanol was added before the enzyme. With moccasin venom, the ester:P ratio decreased from 1·89 to 1·00 in about 2 hr. Fig. 1 shows the time course of the reaction, the mean values being

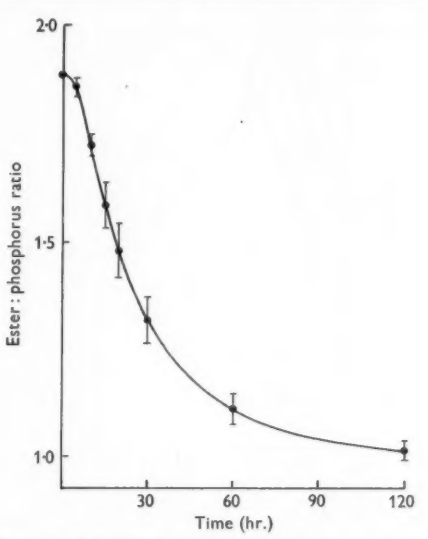

Fig. 1. Time course of enzymic hydrolysis of ovolecithin, prepared according to Pangborn (1951), by moccasin-venom phospholipase *A*. Reaction mixtures contained 1·5–2·0 μmoles of ovolecithin in 1 ml. of dry peroxide-free ether, and 10 μg. of venom in 0·01 ml. of 0·005 M-$CaCl_2$. Temp., 18°. Mean values ± S.E. based on twelve experiments.

based on 12 experiments, with the indicated standard error of the mean. It appears that there is a very noticeable time lag of about 5 min. in the enzymic degradation. Qualitatively similar results were obtained with rattlesnake and cobra venom, although their activities were somewhat lower than that of moccasin venom.

Effect of calcium ions. The presence of Ca^{2+} ions at certain concentrations increased the rate of degradation of ovolecithin by moccasin-venom phospholipase *A*. The optimum concentration of Ca^{2+} ions was found to be independent of the amount of venom used, but varied with the concentration of ovolecithin, as shown in Fig. 2. Optimum final concentrations of Ca^{2+} ions (in the ethereal solution) for 1·3, 2·0 and 3·3 μmoles of ovolecithin/ml. were about 40, 50 and 80 μM respectively. These concentrations are based on the assumption that the Ca^{2+} ions, introduced with the venom solution, are distributed throughout the moist ethereal phase. This seems to be so in the early stages of the reaction. However, it has been found in a large-scale experiment that at the end of the reaction the Ca^{2+} ions are no longer present in the ethereal solution. Ovolecithin (75 mg.), dissolved in ether (50 ml.), was treated with 0·5 ml. of 0·1% moccasin venom in 0·005 M-$CaCl_2$. After 2 hr. at room temperature, the precipitated lysolecithin was removed by centrifuging and the ethereal supernatant was evaporated to dryness and incinerated. No calcium could be detected in the residue, although 0·05 ml. of the venom solution, on analysis by an oxalate method, was found to contain 92 μg. of calcium (theoretical, 100 μg.).

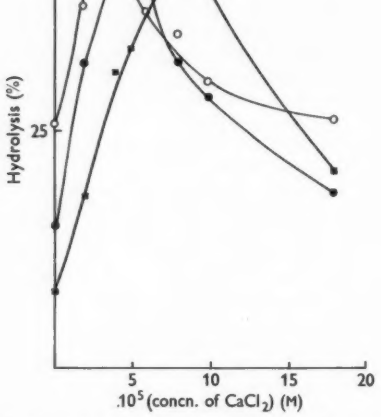

Fig. 2. Dependence of optimum concn. of Ca^{2+} ions on concn. of ovolecithin. Separate samples of venom (1 mg.) were dissolved in 1 ml. of aqueous $CaCl_2$, having 100 times the concn. shown on the abscissa, and 0·01 ml. portions were added to 1·0 ml. of dry peroxide-free ether containing ovolecithin. Concns. of ovolecithin (μmoles/ml.): ○, 1·3; ●, 2·0; ■, 3·3. Reaction stopped after 45 min. at room temp. (18°). Removal of one ester group/mole of ovolecithin is represented by 50% hydrolysis.

That Ca^{2+} ions are probably essential for phospholipase *A* activity is shown by the inhibition of the reaction by ethylenediaminetetraacetic acid (Table 1).

The following metal chlorides were tested for their ability to replace $CaCl_2$, but were found lacking in activity: NaCl, KCl, $BaCl_2$, $SrCl_2$, $MgCl_2$ and $CdCl_2$. Inhibition was observed with $CuCl_2$ and $ZnCl_2$.

Effect of inhibitors. Ethylenediaminetetraacetic acid inhibited moccasin-venom phospholipase *A* activity, presumably by its ability to form a complex with Ca^{2+} ions (Table 1). Inhibition (95%) by mM-Cu^{2+} ions suggested that the active centre of the enzyme might contain a sulphydryl group. However, at final concentration mM, neither sodium iodoacetate nor sodium *p*-chloromercuribenzoate had any inhibitory action and the enzyme was not activated by mM sodium mercaptoacetate (thioglycollate). With regard to inhibitors, this enzyme closely resembles the phospholipase *B* of rat liver (Dawson, 1956). There seems little evidence for the presence of a sulphydryl group in the active centre of phospholipase *A*.

Action of moccasin-venom phospholipase A *on saturated lecithins*

Since those lecithins which contain only saturated fatty acid radicals are sparingly soluble in pure ether, mixtures of other solvents with ether were tested for their effects on phospholipase *A* activity.

Table 1. *Effect of ethylenediaminetetraacetic acid on moccasin-venom phospholipase* A *activity*

To a series of tubes, each containing about 2·0 μmoles of ovolecithin in 1 ml. of ether, was added 0·01 ml. of 0·1% venom containing $CaCl_2$ and ethylenediaminetetraacetic acid (EDTA) in the concentrations shown. Incubation for 30 min. at 18°.

Concn. of $CaCl_2$ in venom (mM)	Concn. of EDTA in venom (mM)	Hydrolysis (%)
0	0	38
0	1	0
0	2	0
3·5	0	45
3·5	1	27
3·5	2	0

With ovolecithin as substrate, ether–dioxan (2:1, v/v), ether–$CHCl_3$ (19:1, v/v) and ether–ethanol (99:1, v/v) had virtually no inhibitory effect when compared with pure ether. Ether–ethanol (19:1, v/v), however, inhibited completely. The experiments with saturated lecithins were carried out either in ether–ethanol or ether–$CHCl_3$ (both 99:1, v/v), in which solvents they were soluble.

Fig. 3 shows the rates of enzymic degradation of some natural and synthetic saturated lecithins, compared with that of ovolecithin. The hydrolysis of natural dipalmitoyl-L-α-lecithin (and also of synthetic dimyristoyl-L-α-lecithin, not shown in Fig. 3) was more rapid than that of ovolecithin. The degradation of synthetic dipalmitoyl-DL-α-lecithin (and of distearoyl-DL-α-lecithin, not shown in Fig. 3) took place more slowly than that of ovolecithin and the reaction stopped when 0·5 μmole of ester/μg.atom of phosphorus had been removed. Synthetic dipalmitoyl-β-lecithin was not attacked.

Hydrogenated ovolecithin was also attacked by phospholipase *A* though less rapidly than was ovolecithin; it will be shown later (Fig. 7) that the rate of degradation of hydrogenated ovolecithin could be increased by raising the pH of the ethereal solution.

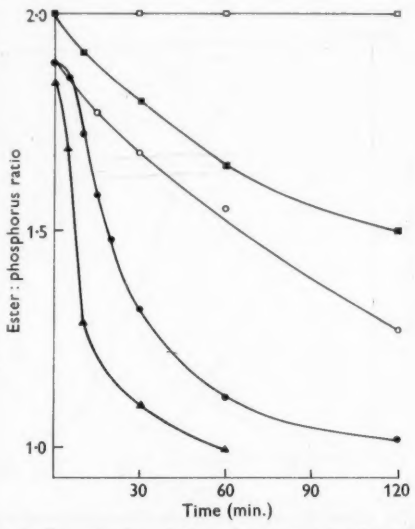

Fig. 3. Enzymic degradation of saturated lecithins. The substrates (about 20 μmoles) were dissolved in ethanol (0·1 ml.) and diluted to 10 ml. with ether. Samples (1 ml.) were treated with 0·01 ml. of 0·1% moccasin venom in 0·005 M-$CaCl_2$. □, Dipalmitoyl-β-lecithin (synthetic); ■, dipalmitoyl-DL-α-lecithin (synthetic); ▲, dipalmitoyl-L-α-lecithin (natural); ○, hydrogenated ovolecithin; ●, ovolecithin (for comparison).

The enzymic hydrolysis of dimyristoyl-L-α-lecithin was stimulated by Ca^{2+} ions.

Action of moccasin-venom phospholipase A on 'kephalins'

The literature contains conflicting statements concerning the action of snake-venom on 'kephalins'. Levene & Rolf (1923) showed that ethanolamine-containing phospholipids, present in a phosphate-buffered egg-yolk emulsion at pH 7·5, were attacked by snake venom, and Levene, Rolf & Simms (1924) isolated both lysolecithin and 'lysokephalin' from such a reaction mixture. However, Chargaff & Cohen (1939) reported that snake venom was without effect on aqueous emulsions of several partially purified kephalins.

In preliminary experiments, using the technique of Hanahan *et al.* (1954) modified by the addition of Ca^{2+} ions, we were able to demonstrate the breakdown in ethereal solution of crude ox-brain kephalin fractions by moccasin venom. These substrates were virtually free from choline-containing material, and consisted of a mixture of phosphatidylethanolamine and phosphatidylserine, grossly contaminated with plasmalogens. Their rates of enzymic degradation were rather slow compared with that of ovolecithin, as shown in Fig. 4. Nevertheless, after

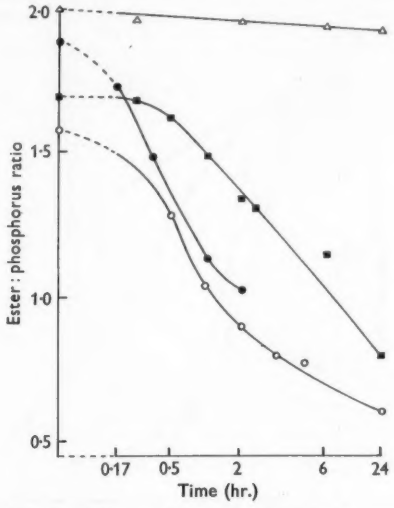

Fig. 4. Degradation of kephalins by moccasin venom. Conditions as in Fig. 3. ■, Ox-brain kephalin, 'fractions III–V' (Folch, 1949); ○, an ethanol-insoluble fraction of ox brain (see Methods section); △, pure egg phosphatidylethanolamine (Lea *et al.* 1955); ●, ovolecithin (for comparison). The abscissa has been drawn to a logarithmic scale.

24 hr. these substrates had lost almost exactly one ester group/atom of phosphorus.

When pure egg phosphatidylethanolamine, prepared by silica-gel chromatography (Lea *et al.* 1955), was used as substrate, however, no enzymic degradation could be detected (Fig. 4). Furthermore, addition of a small quantity of pure egg phosphatidylethanolamine to ovolecithin strongly inhibited the enzymic degradation of the latter (Fig. 5). The lag phase was also greatly prolonged, a finding which suggested that the short lag phase previously found with ovolecithin, prepared by the Pangborn (1951) procedure (Fig. 1), could have been due to the presence of a small amount of phosphatidylethanolamine as an impurity. However, when ovolecithin, free from phosphatidylethanolamine, prepared by the chromatographic method of Lea *et al.* (1955), was tested, its rate of enzymic degradation by moccasin venom was practically zero (see Fig. 6). An explanation of these results is given in the next section.

Effect of pH *on phospholipase* A *activity*

In the experiments of Levene & Rolf (1923) aqueous emulsions of egg yolk, buffered with phosphate to pH 7·5, were treated with snake venom. Hanahan (1952), when first reporting the increased activity of phospholipase *A* in ethereal solution, buffered his aqueous enzyme–lecithin reaction mixture to pH 7·0 with glycylglycine before extracting with ether. In subsequent work, however, Hanahan *et al.* (1954) did not buffer the reaction mixture, since the enzymic hydrolysis took place in ether–water (99:1, v/v) where pH was considered to have little significance. In the experiments so far described, no buffers were used.

The observation that small amounts of pure phosphatidylethanolamine strongly inhibited the degradation of ovolecithin by snake-venom phospholipase *A* (Fig. 5) raised the possibility that inhibition might also be obtained in the presence of ethanolamine itself. However, when 0·5 μmole of the free base was added to 1 ml. of an ethereal solution of ovolecithin (2 μmoles), the rate of degradation of the latter by moccasin venom was significantly increased. Ethanolamine acetate, by contrast, had no such stimulating effect. This suggested that the increased enzymic activity was caused by a rise in pH of the ethereal solution, support for which was obtained by using ammonia in place of ethanolamine (see Fig. 8). Other experiments, in which the enzyme was first dissolved in water-soluble buffers, in an attempt to control the pH of the ethereal reaction mixture, proved unsuccessful since the ether either did not dissolve the buffer at all or else preferentially dissolved one of

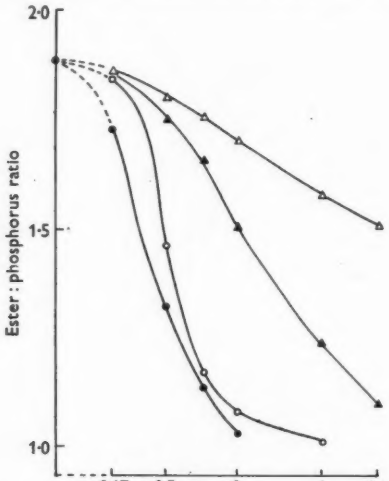

Fig. 5. Inhibition of enzymic degradation of ovolecithin by pure egg phosphatidylethanolamine. All ethereal reaction mixtures (1 ml.) contained 2 μmoles of ovolecithin. Amount of added phosphatidylethanolamine (μmoles): ●, Nil; ○, 0·01; ▲, 0·02; △, 0·10.

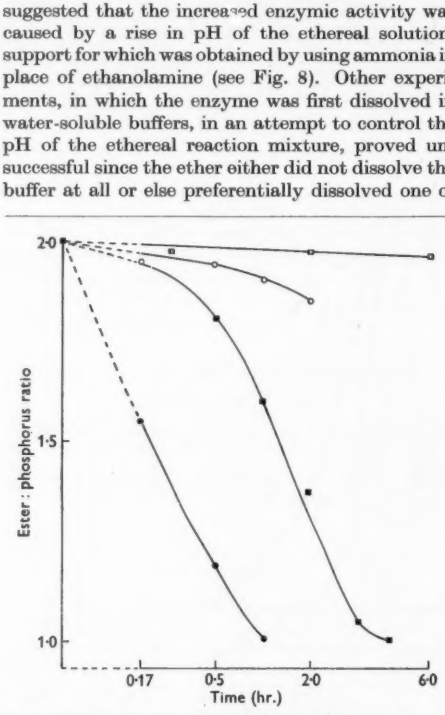

Fig. 6. Effect of ammonia on the degradation of ovolecithin and egg phosphatidylethanolamine, prepared chromatographically (Lea *et al.* 1955), by snake-venom phospholipase *A*. Substrates (2 μmoles) were dissolved in 1 ml. of ether; enzyme, moccasin venom (0·01 ml. of a 0·1% solution in aqueous 0·005 M-$CaCl_2$). With lecithin: ○, no addition; ●, 0·5 μmole of ammonia present. With phosphatidylethanolamine: □, no addition; ■, 0·5 μmole of ammonia present.

the buffer constituents, e.g. succinic acid from a succinate buffer. In subsequent experiments, therefore, ammonia, which is very soluble in ether, was used exclusively for adjusting the pH of the ethereal reaction mixture.

When 0·5 μmole of ammonia was added to an ethereal solution (1 ml.) of pure egg phosphatidylethanolamine (2 μmoles), the latter was also degraded by snake-venom phospholipase *A*, reaction being complete in less than 6 hr. at room temperature (Fig. 6). When 1·5 μmoles of ammonia were added, the stimulation of enzymic activity was rather less, presumably because the reaction mixture now had a pH higher than the optimum.

It is difficult to determine accurately the pH of the ethereal reaction mixtures by means of indicators. However, it has been found empirically that the optimum pH for enzymic activity could be obtained in the following way. One drop of an ethereal solution of bromocresol green (0·1 %) was added to an ethereal solution of the phosphoglyceride, and an ethereal solution of ammonia (50 μmoles/ml.) was added until the tint matched that of the same indicator in an aqueous buffer of pH 5·2. It has been observed, however, that the pH ranges of bromocresol green in aqueous solution and in ethereal solution are different, and that the value of 5·2 is only the apparent pH optimum. Thus, when the pH of such an ethereal solution was determined with a pH meter and glass electrode assembly, a stable reading of 7·2 was obtained. Further comparisons between the apparent pH of other ethereal solutions, as estimated by their colours with bromocresol green, and the true pH values, as determined with a pH meter, showed the latter to be consistently about 2 pH units higher. Thus it appears that the pH optimum for moccasin-venom phospholipase *A* activity in ethereal solution is about 7·2, a value which is within the range found for aqueous emulsions by Levene & Rolf (1923) and Hanahan (1952).

For convenience in estimating the pH of ethereal solutions of the phosphoglycerides, the colorimetric method using bromocresol green has been applied and the values obtained corrected by adding 2 pH units. In this way, an ethereal solution of ovolecithin, prepared by Pangborn's (1951) procedure, was found to have a pH of 6·6, while ovolecithin and egg phosphatidylethanolamine, prepared by silica-gel chromatography (Lea *et al.* 1955), had pH values below 6·0; addition of the required amount of ammonia to 2 μmoles of any one of these substrates in 1 ml. of ether in order to raise the pH to 7·0–7·2, caused a rapid hydrolysis by moccasin-venom

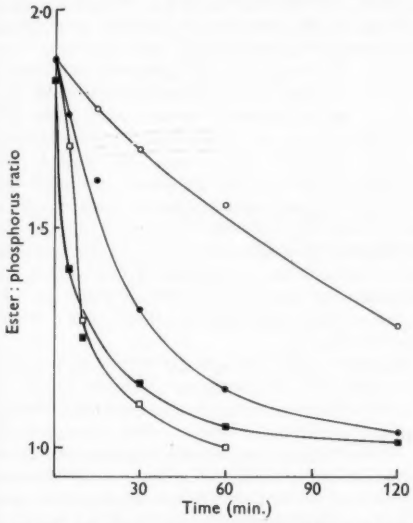

Fig. 7. Effect of ammonia on the degradation of hydrogenated natural lecithins by snake-venom phospholipase *A*. Experimental conditions as in Fig. 6. Hydrogenated ovolecithin (original pH 6·0–6·2): ○, no addition; ●, 0·5 μmole of ammonia present. Dipalmitoyl-L-α-lecithin (Hanahan *et al.* 1954; original pH 7·0–7·2): □, no addition; ■, 0·5 μmole of ammonia present.

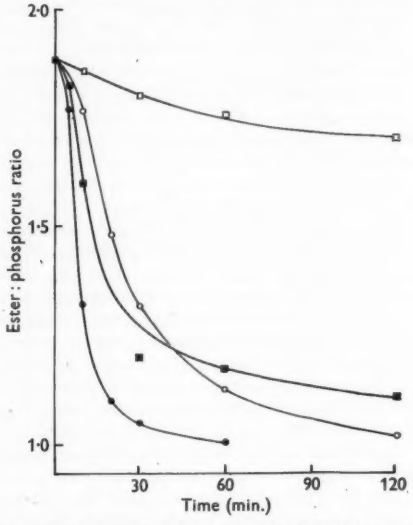

Fig. 8. Effect of ammonia on the inhibition of the enzymic degradation of ovolecithin by egg phosphatidylethanolamine. Experimental conditions as in Fig. 6. All reaction mixtures contained 2 μmoles of ovolecithin, prepared according to Pangborn (1951). ○, No addition; ●, 0·05 μmole of ammonia; □, phosphatidylethanolamine (0·1 μmole); ■, phosphatidylethanolamine and ammonia.

phospholipase *A* (Fig. 6). Hydrogenated ovolecithin, the slow breakdown of which has already been mentioned (Fig. 3), had a pH in ethereal solution of 6·0–6·2; its enzymic degradation was likewise stimulated by the addition of ammonia (Fig. 7), although even at the optimum pH it was not hydrolysed as rapidly as ovolecithin. On the other hand, the sample of dipalmitoyl-L-α-lecithin, prepared by catalytic hydrogenation of yeast dipalmitoleyl-L-α-lecithin (Hanahan & Jayko, 1952), which in turn had been eluted from an alumina column, had a pH of 7·0–7·2, and its rate of enzymic degradation was not increased by addition of ammonia (Fig. 7).

The inhibition of the enzymic degradation of ovolecithin by egg phosphatidylethanolamine may partly be attributed to its action in lowering the pH of the ethereal reaction mixture. This is shown by the experiment in Fig. 8, in which addition of ammonia almost abolishes this inhibition. The mixture of ovolecithin (2 μmoles) and phosphatidylethanolamine (0·1 μmole) had a pH in ethereal solution of 6·2, which was raised to 7·0 in the presence of 0·5 μmole of ammonia.

Neither diphosphoinositide (Folch, 1949) nor phosphatidylinositol (Hawthorne, 1955) was attacked by phospholipase *A*, even in the presence of ammonia.

DISCUSSION

Requirement for calcium ions

The stimulation of moccasin-venom phospholipase *A* activity by calcium ions (Fig. 2) has not previously been reported, so far as we are aware, although Zeller (1952) carried out the enzymic degradation of synthetic dimyristoyl-L-α-lecithin in the presence of 5×10^{-4} M-Ca^{2+} without commenting on this addition. It may also be significant that Ca^{2+} ions were found by Braganca & Quastel (1953) to increase the inhibition of glucose oxidation in rat-brain homogenate caused by cobra venom. Ca^{2+} ions stimulated the phospholipase *A* activity of all eight venom samples tested and increased the rate of enzymic degradation of all the substrates examined except the dipalmitoyl-L-α-lecithin of Hanahan *et al.* (1954).

It is of interest that the optimum requirement for Ca^{2+} ions should depend upon the concentration of lecithin used. It has been calculated from the data in Fig. 2 that each μmole of ovolecithin requires about 0·03 μmole of Ca^{2+} ions for maximal rate of hydrolysis. This might suggest that the Ca^{2+} ions acted by combining with some inhibitor present in the ovolecithin were it not for the fact that the synthetic lecithins also require Ca^{2+} ions for full activity. The absolute necessity for Ca^{2+} ions is also supported by the observed inhibitory effect of ethylenediaminetetraacetic acid on phospholipase *A* activity (Table 1) and the fact that Ca^{2+} ions could not be replaced by other bivalent cations. The possibility that Ca^{2+} ions act by combining with the liberated fatty acids can also be excluded by the observation that only 0·03 μmole of Ca^{2+} ions is required for each μmole of fatty acid liberated and that the Ca^{2+} ions are carried out of solution by the precipitated lysolecithin, whereas the fatty acids remain in the ethereal supernatant. The most likely explanation is that the Ca^{2+} ions take part in the binding of the enzyme with the substrate, since Hanahan *et al.* (1954) have shown that the enzyme is also carried out of solution by the precipitated lysolecithin and we have found that when reaction is incomplete, the precipitated lysolecithin adsorbs unchanged lecithin from the ethereal solution.

Substrate specificity of phospholipase A

The results of experiments with synthetic lecithins leave no room for doubt that phospholipase *A* is specific for the L-α-structure. Synthetic dimyristoyl-L-α-lecithin and the naturally occurring lecithins, which also possess the L-α-structure (Long & Maguire, 1953, 1954), are hydrolysed with the removal of one ester group per molecule. The nature of the fatty acid radicals does not seem to be very important, provided that a small quantity of ethanol or chloroform is present so as to bring the saturated lecithins into ethereal solution, and that the pH is in the region of 7·0. Synthetic DL-α-lecithins, on the other hand, lose one ester group/two molecules of substrate, which strongly suggests that the D-α form is not attacked. A synthetic β-lecithin was not degraded by phospholipase *A*.

Pure egg phosphatidylethanolamine has also been found to act as a substrate for phospholipase *A* when the pH of the ethereal solution was adjusted to about 7·0. It seems probable that phosphatidylethanolamine has a higher affinity than lecithin for the active centres of the enzyme, for otherwise it is difficult to account for the slower rate of attack on a mixture of ovolecithin and phosphatidylethanolamine (95:5) than on ovolecithin alone, even when the pH has been correctly adjusted (Fig. 8).

Whether other phosphoglycerides can act as substrates for phospholipase *A* must remain in doubt until pure samples become available. Present evidence indicates, however, that phosphatidylserine and ethanolamine plasmalogen are hydrolysed (Fig. 4), while Franzl & Rapport (1955) have stated in a preliminary report that choline plasmalogen (phosphatidalcholine) was degraded by snake-venom phospholipase *A*, although the ethanolamine analogue was not. Neither of the inositol phosphoglycerides was attacked by phospholipase *A*. Perhaps it is necessary that the substrate molecule should possess a positively charged group, as in lecithin and phosphatidylethanolamine.

SUMMARY

1. The enzymic degradation of ovolecithin and certain other phosphoglycerides in ethereal solution by snake-venom phospholipase *A* is stimulated by Ca^{2+} ions. The optimum Ca^{2+} ion concentration varies between 40 and 80 μM, when the lecithin concentration varies between 1·3 and 3·3 mM.

2. The phospholipase *A* activity of moccasin venom is inhibited by ethylenediaminetetraacetic acid and by Zn^{2+} and Cu^{2+} ions, but not by iodoacetate or *p*-chloromercuribenzoate.

3. All the natural and synthetic L-α-lecithins studied lost one ester group/molecule of substrate in the presence of the enzyme. Synthetic DL-α-lecithins lost only 0·5 mole of ester/molecule of substrate, whereas synthetic β-lecithins did not undergo any enzymic hydrolysis.

4. Egg phosphatidylethanolamine was degraded by phospholipase *A* when the ethereal solution was adjusted to pH 7·0, and there is some evidence for the breakdown of phosphatidylserine and ethanolamine plasmalogen.

5. Inositol phosphoglycerides were not attacked by moccasin-venom phospholipase *A*.

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Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

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There is no need to stress the potential value in many connexions of a class of selective inhibitors for the different glycosidases. Levvy (1952) discovered that mouse-liver β-glucuronidase is very powerfully inhibited by saccharo-1:4-lactone, and the presence of this lactone in traces in saccharate solutions explained the powerful inhibitory action of the latter on this enzyme (Karunairatnam & Levvy, 1949). Saccharo-1:4-lactone, as such, or in the form of a saccharate solution, has been shown to inhibit β-glucuronidase from every source that has been studied. Conchie (1954) found that β-glucosidase from rumen micro-organisms was inhibited by the 1:4- and 1:5-lactones of gluconic acid, but not by saccharo-1:4-lactone. These findings have been confirmed with β-glucosidase from other sources (Jermyn, 1955; Robinson, 1956). Although efficient inhibitors, the gluconolactones are not as powerful as saccharo-1:4-lactone.

It was decided to extend these studies to other types of glycosidase, and at the same time examine the specificity of response to a range of lactones. This work also served another purpose in that rumen liquor was screened for enzymes hydrolysing

the glycosides of some of the commoner sugars. Since rumen liquor was deficient in some of these enzymes, they had to be prepared from other sources, and attention was ultimately turned to the common limpet (*Patella vulgata*), which displayed an astonishing range of glycosidase activity. Limpet preparations hydrolysed all the glycosides employed in the present work, and several others besides (Conchie, Levvy & Marsh, 1956).

EXPERIMENTAL

Materials

All melting points are corrected.

p-Nitrophenyl α-D-glucoside, prepared as described by Montgomery, Richtmyer & Hudson (1942), had the constants recorded by them: m.p. 216°, $[\alpha]_D^{17}$ +215° in water (*c*, 1·0).

o-Nitrophenyl β-D-glucoside, prepared as described for the galactoside (Seidman & Link, 1950), had m.p. 152°, $[\alpha]_D^{16}$ −107° in water (*c*, 1·2); Montgomery *et al.* (1942) give the same constants.

Phenyl β-D-glucoside, prepared as described by Montgomery *et al.* (1942), had m.p. 175°, $[\alpha]_D^{16}$ −71° in water (*c*, 1·0); Fischer & von Mechel (1916) give m.p. 175–176°, $[\alpha]_D$ −72°.

Phenyl α-D-galactoside, prepared as described for the glucoside (Montgomery *et al.* 1942), had m.p. 145°, $[\alpha]_D^{16}$ +215° in water (*c*, 1·4); Helferich & Appel (1932) give m.p. 146°, $[\alpha]_D$ +217°.

o-Nitrophenyl β-D-galactoside, prepared as described by Seidman & Link (1950), had m.p. 193°, $[\alpha]_D^{17}$ −51° in water (*c*, 1·0); Seidman & Link (1950) give m.p. 193–194°, $[\alpha]_D$ −52°.

Phenyl α-D-mannoside, prepared as described by Helferich & Winkler (1933), had m.p. 131–132°, $[\alpha]_D^{17}$ +113° in water (*c*, 1·0); Helferich & Winkler (1933) give m.p. 132–133°, $[\alpha]_D$ +114°.

Phenyl β-D-mannoside, prepared as described by Helferich & Winkler (1933), had m.p. 174–175°, $[\alpha]_D^{15}$ −71° in water (*c*, 0·9); Helferich & Winkler (1933) give m.p. 175–177°, $[\alpha]_D$ −72°.

Phenyl β-D-xyloside, prepared as described by Montgomery *et al.* (1942), had m.p. 178–179°, $[\alpha]_D^{15}$ −48° in water (*c*, 0·9); Montgomery *et al.* (1942) give m.p. 178–180°, $[\alpha]_D$ −49°.

Phenolphthalein β-D-glucuronide was prepared biosynthetically (Talalay, Fishman & Huggins, 1946).

Phenyl *N*-acetyl-α-D-glucosaminide: the tetra-*O*-acetyl derivative was prepared as described by Roseman & Dorfman (1951) and deacetylated with barium methoxide; m.p. 244–246°; Roseman & Dorfman give m.p. 246–247°.

Phenyl *N*-acetyl-β-D-glucosaminide: the tetra-*O*-acetyl derivative was prepared as described by Helferich & Iloff (1933), the fusion being continued for 5 hr., and deacetylated with barium methoxide; m.p. 248–249°; Roseman & Dorfman (1951) give m.p. 249–250°.

D-Glucono-1:5-lactone (British Drug Houses Ltd.) was twice recrystallized from ethanol; m.p. 153°, $[\alpha]_D^{18}$ +63° in water (*c*, 1·8): Hedenburg (1915) gives the same constants.

D-Glucono-1:4-lactone, prepared as described by Hedenburg (1915) from calcium gluconate, had m.p. 135°, $[\alpha]_D^{18}$ +67° in water (*c*, 1·8): Hedenburg (1915) gives the same constants.

D-Mannono-1:4-lactone, prepared as described by Nelson & Cretcher (1930), had the constants recorded by them: m.p. 151–152°, $[\alpha]_D^{15}$ +51° in water (*c*, 1·0).

D-Arabono-1:4-lactone (L. Light and Co. Ltd.) was twice recrystallized from acetone; m.p. 95–96°, $[\alpha]_D^{15}$ +71° in water (*c*, 1·0); Jensen & Upson (1925) give m.p. 96°, $[\alpha]_D$ +73°.

Saccharo-3:6-lactone, prepared as described by Smith (1944), had m.p. 146°, $[\alpha]_D^{15}$ +42° in water (*c*, 1·0); Smith (1944) gives m.p. 149°, $[\alpha]_D$ +45°.

D-Galacturonic acid (Eastman Kodak Co.) was twice recrystallized from 90% ethanol; m.p. 158–159°, $[\alpha]_D^{15}$ +54° in water (*c*, 1·0); Link & Dickinson (1930) give m.p. 159–160°, $[\alpha]_D$ +54°.

Racemic acid (British Drug Houses Ltd.) was twice recrystallized from water to remove *meso*-tartaric acid; m.p. 203–204°; Bischoff & Walden (1889) give the same m.p.

The following commercial products were used without purification: D-, L- and *meso*-tartaric acids (British Drug Houses Ltd.); D-galactono-1:4-lactone, L-gulono-1:4-lactone, D-ribono-1:4-lactone and α-D-glucoheptono-1:4-lactone (L. Light and Co. Ltd.); D-glucurono-3:6-lactone (Corn Products Refining Co.).

The following compounds were gifts: L-galactono-1:4-lactone (Dr W. W. Reid), saccharo-1:4-lactone (Professor F. Smith), xylono-1:4-lactone (Dr H. S. Isbell), D-mannurono-3:6-lactone (Dr C. A. Marsh).

Saccharate solutions were prepared from potassium hydrogen saccharate (British Drug Houses Ltd.) and the solutions were boiled before use for 30 min. at acid pH to cause one-third conversion into saccharo-1:4-lactone (Levvy, 1952).

Mucic acid (British Drug Houses Ltd.) was thrice recrystallized from water; m.p. 219°: figures varying from 206° to 225° are given in the literature. Before use, solutions were boiled for 1 hr. to cause maximum lactonization (Levvy, 1952).

Enzyme preparations

From rumen liquor. Cheviot sheep with permanent rumen fistulae were kept on a diet of 500 g. of hay, supplemented with 450 g. of grass cubes at 7 a.m. and again at 4 p.m., and with free access to water and salt lick. Rumen liquor withdrawn at 9.30 a.m. was strained through nine layers of surgical gauze and centrifuged at 10000 *g* for 20 min. The deposit was washed on the centrifuge with 1% (w/v) NaCl solution and suspended in water (½ vol. of rumen liquor). Micro-organisms were broken down by shaking with grade 12 Ballotini beads (Chance Bros. Ltd.) in the Mickle disintegrator (Mickle, 1948) for 45 min. The suspension was centrifuged at 10000 *g* for 30 min. and the cell-free supernatant was brought to pH 5·2 and 20% saturation with ammonium sulphate. After 30 min. at 0°, the precipitate was centrifuged off at 1500 *g* for 15 min., and the supernatant brought to 80% saturation with ammonium sulphate. The precipitate that formed after 30 min. at 0° was centrifuged off at 10000 *g* for 30 min. and dissolved in water. The final volume of the enzyme preparation was arranged to give a suitable liberation of aglycone in the enzyme assay (140–180 μg. of *o*-nitrophenol, 90–130 μg. of *p*-nitrophenol, 40–50 μg. of phenol), and was usually about 10% of the original volume of rumen liquor. Samples of rumen liquor are of course very variable. The enzyme blank

in all assays by the phenol method was reduced to negligible proportions by the ammonium sulphate fractionation.

From lucerne seed. Lucerne seed (De Puits, France) was suspended in water (60 ml./g. of seed) with a glass homogenizer. After centrifuging off insoluble material at 10000 *g* for 30 min., the preparation was brought to pH 5·2 and fractionated with ammonium sulphate as above. The final fraction was dissolved in 60 ml. of water/g. of seed.

From limpets. Freshly dissected limpet 'visceral humps' were homogenized in water (10 ml./g. of moist tissue), and after freezing and thawing were freed from insoluble material by centrifuging at 10000 *g* for 15 min. Fractionation with ammonium sulphate was done as described above, and the final fraction dissolved in 2·5 ml./g. of tissue. This stock solution was kept at −20° and diluted as required, according to the enzyme to be studied. Dilutions of the stock solution were approximately as follows: α-glucosidase, 1:5; β-glucosidase, 1:40; α-galactosidase, 1:7; β-galactosidase, 1:20; α-mannosidase, 1:5; β-mannosidase, 1:10; α-*N*-acetyl-glucosaminidase, 1:4; β-*N*-acetyl-glucosaminidase, 1:5; β-xylosidase, 1:7; β-glucuronidase, 1:400.

From mouse liver. Mouse-liver β-glucuronidase was prepared as described by Levvy (1952).

Enzyme assay

Liberation of o- *and* p-*nitrophenol.* Optimum hydrolysis of *o*-nitrophenyl β-glucoside by a rumen preparation in phosphate–citrate buffer was found to occur at pH 5·4 and a substrate concentration of 5 mM (Conchie, 1954). The incubation mixture contained 0·5 ml. of enzyme preparation in a final volume of 4 ml., and the colour of the free aglycone was developed by addition of glycine–Na_2CO_3 buffer. The same procedure was arbitrarily adopted for other *o*- or *p*-nitrophenyl glycosides and enzyme preparations. In some instances, however, the substrate concentration was reduced to 2·5 mM.

Liberation of phenol. Hydrolysis of phenyl glycosides was followed by the procedure of Kerr, Graham & Levvy (1948), with Ilford no. 608 red filter in the Spekker absorptiometer to measure free phenol. Incubation of enzyme with substrate was carried out for 1 hr. at 37° in 0·05M citrate buffer. The pH was arbitrarily fixed at 5·2 and the substrate concentration at 2·5 or 5 mM. The incubation mixture contained 0·4 ml. of enzyme preparation in a final volume of 1 ml. Suitable corrections were made for interference by uronic acids (Karunairatnam & Levvy, 1949).

Liberation of phenolphthalein. The liberation of phenolphthalein from 0·625 mM phenolphthalein β-glucuronide by mouse-liver β-glucuronidase in 0·125M acetate buffer, pH 5·2, was measured as described by Levvy (1952). There was 0·5 ml. of enzyme preparation in the incubation mixture, final volume 4 ml. For limpet β-glucuronidase the same conditions were employed, except that the pH was arbitrarily fixed at 4·5.

RESULTS

Table 1 shows the results obtained when all the lactones were screened for inhibitory power at a concentration of 10 mM against α- and β-glucosidase, galactosidase and mannosidase, as well as β-xylosidase and β-glucuronidase. To conserve pure saccharo-1:4-lactone, boiled saccharate solution was used in these tests, and boiled mucate solution was employed in place of the unknown galactosaccharolactone. Galacturonic acid (unboiled) was used as such since it does not form a lactone. Preliminary experiments with rumen preparations stressed the need to work with cell-free extracts; otherwise, inconsistent results were obtained, no doubt owing to permeability and other factors.

Rumen liquor was found to lack α- and β-mannosidase activity. The former enzyme was therefore obtained from lucerne seed (Hill, 1934), and the opportunity was taken to examine the α-galactosidase also present in the preparation (Hill, 1934). Traces of β-mannosidase activity have been reported in baker's and brewer's yeast (Adams, Richtmyer & Hudson, 1943), but we were unable to confirm this observation with the yeast strains at our disposal. A β-mannosidase has been described in the snail *Helix laeda* Gould (Nagaoka, 1949), and the enzyme was therefore sought in the more readily available mollusc, *Patella vulgata*, with successful results. Since *P. vulgata* is rich in β-glucuronidase activity (Dodgson, Lewis & Spencer, 1953), this enzyme was included in these tests. Mouse-liver β-glucuronidase was examined too, in preference to the similar enzyme in rumen liquor (Karunairatnam & Levvy, 1951).

The limpet preparation was found to possess all the enzymes of present interest, and most of the lactones already found to be inhibitory were retested at 10 mM with this preparation. Also included in these experiments were limpet α- and β-*N*-acetyl-glucosaminidase. The results are shown in Table 2, with the exception of the figures for β-mannosidase and β-glucuronidase, these enzymes being more fully dealt with in Table 1.

Above 70% inhibition of enzyme activity, quantitative comparison of different inhibitors is not possible because the curve relating percentage inhibition to inhibitor concentration is asymptotic. All those lactones which were powerful inhibitors of at least one enzyme were therefore retested at concentrations which gave marked, but not excessive, inhibition. Table 3 shows the comparative effects of the inhibitors at these concentrations on the different enzymes. Saccharate solution was now replaced by pure saccharo-1:4-lactone, and mucate was omitted.

One factor that is diminished in the experiments in Table 3 is the effect of trace impurities in the lactone solutions. Thus to take an extreme case, 0·025% saccharo-1:4-lactone present as impurity in any other lactone will lead to nearly 50% inhibition of β-glucuronidase by a 10 mM solution of the latter. There is no doubt that the presence of saccharate explains the feeble inhibitory action of gluconate on β-glucuronidase under conditions that permit lactonization (Karunairatnam & Levvy, 1949;

Table 1. *Percentage inhibition of glycosidases from various sources by sugar acid lactones in a final concentration of* 10 *mM*

Sources of enzymes: mixed rumen micro-organisms; lucerne seed; limpet visceral hump; mouse liver. Assay by phenol, nitrophenol and phenolphthalein methods. Results are expressed as percentages of controls.

	Inhibition (%)											
	p-Nitrophenyl α-glucoside (2·5 mM)	*o*-Nitrophenyl β-glucoside (5 mM)	Phenyl β-glucoside (5 mM)	Phenyl α-galactoside (5 mM)		*o*-Nitrophenyl β-galactoside (2·5 mM)	Phenyl α-mannoside (5 mM)	Phenyl β-mannoside (2·5 mM)	Phenyl β-xyloside (5 mM)	Phenolphthalein β-glucuronide (0·625 mM)	
Lactone	Rumen	Rumen	Rumen	Rumen	Lucerne	Rumen	Lucerne	Limpet	Rumen	Limpet	Mouse liver
α-D-Glucoheptono-1:4-	0	2	5	2	−2	0	0	0	7	0	0
D-Glucono-1:5-	73	95	99	7	−1	0	30	2	27	0	0
D-Glucono-1:4-	75	93	97	0	1	0	23	2	17	0	0
D-Galactono-1:4-	0	7	7	2	33	82	2	1	0	0	0
L-Galactono-1:4-	0	5	0	4	−1	2	2	2	0	0	0
D-Mannono-1:4-	1	4	10	4	−2	1	78	92	0	0	0
L-Gulono-1:4-	2	0	0	3	0	2	1	1	21	0	0
D-Arabono-1:4-	3	22	28	4	0	0	8	41	17	0	1
D-Xylono-1:4-	2	7	1	4	0	14	2	2	72	0	5
D-Ribono-1:4-	2	5	6	4	4	0	0	0	6	0	1
D-Glucurono-3:6-	0	6	0	2	0	1	2	0	0	0	2
D-Mannurono-3:6-	1	2	0	0	0	1	0	0	0	0	1
Saccharo-3:6-	0	16	6	6	−2	−2	0	2	8	69*	56*
Saccharate (boiled for 30 min.)	1	11	8	1	−3	4	1	0	0	49†	40†
Mucate (boiled for 1 hr.)	0‡	0‡	6‡	0‡	−4‡	0‡	1‡	0‡	0‡	46§	44§
D-Galacturonic acid	0	10	5	0	0	0	2	1	0	12	14

* Final concn. of inhibitor 3 mM.
† Final concn. of inhibitor 0·01 mM.
‡ Final concn. of inhibitor 5 mM (concn. limited by solubility).
§ Final concn. of inhibitor 0·5 mM.

Table 2. *Percentage inhibition of limpet glycosidases by selected sugar acid lactones in a final concentration of* 10 *mM*

Assay by phenol and nitrophenol methods. Results are expressed as percentages of controls.

	Inhibition (%)							
Lactone	*p*-Nitrophenyl α-glucoside (2·5 mM)	*o*-Nitrophenyl β-glucoside (5 mM)	Phenyl α-galactoside (5 mM)	*o*-Nitrophenyl β-galactoside (2·5 mM)	Phenyl α-mannoside (5 mM)	Phenyl β-xyloside (5 mM)	Phenyl *N*-acetyl-α-glucosaminide (3 mM)	Phenyl *N*-acetyl-β-glucosaminide (3 mM)
D-Glucono-1:5-	64	96	5	59	0	92	0	0
D-Glucono-1:4-	71	96	3	61	0	92	0	0
D-Galactono-1:4-	8	33	39	89	0	1	0	0
D-Mannono-1:4-	8	0	0	2	88	3	0	0
D-Arabono-1:4-	18	3	0	2	0	0	0	15
D-Xylono-1:4-	1	24	4	41	1	83	0	0
Saccharate (boiled for 30 min.)	0	0	0	5	5	5	0	0

Table 3. *Effects of selected sugar acid lactones in relatively low concentration on glycosidases from various sources*

For further details, see Table 1.

Substrate	Concn. (mM)	Source of enzyme	Inhibition (%)					
			D-Glucono-1:5-lactone (0·3 mM)	D-Glucono-1:4-lactone (0·3 mM)	D-Galactono-1:4-lactone (0·9 mM)	D-Mannono-1:4-lactone (0·9 mM)	D-Xylono-1:4-lactone (0·9 mM)	Saccharo-1:4-lactone (0·0025 mM)
p-Nitrophenyl α-glucoside	2·5	Limpet	18*	19†	0	0	0	0
o-Nitrophenyl β-glucoside	5	Rumen	59	59	0	0	6	0
		Limpet	64	65	3	0	3	0
Phenyl α-galactoside	5	Limpet	0	0	0	0	0	0
o-Nitrophenyl β-galactoside	2·5	Rumen	0	0	52	1	0	0
		Limpet	59	59	54	0	4	1
Phenyl α-mannoside	5	Limpet	0	0	0	40	0	0
Phenyl β-mannoside	2·5	Limpet	0	0	0	49	0	0
Phenyl β-xyloside	5	Rumen	12	12	0	0	35	0
		Limpet	65	65	−2	−2	48	−2
Phenolphthalein β-glucuronide	0·625	Limpet	0	1	1	1	0	43
		Mouse liver	0	0	0	0	0	34

* 0·9 mM lactone caused 33 % inhibition.

† 0·9 mM lactone caused 32 % inhibition.

Levvy, 1952; Mills, Paul & Smith, 1953); this effect disappears when pure gluconolactones are employed (Table 1; cf. Marsh, 1955). Errors from this source were observed with other enzymes in preliminary experiments with impure lactones.

When it comes to the effects of individual lactones on individual enzymes, it is convenient to consider Tables 1–3 collectively. The most important point that emerges is that, with one exception, all the glycosidases studied, whether α or β, were powerfully inhibited by the aldonolactones with the same arrangement of secondary hydroxyl groups. α-Galactosidase was the exception: in rumen preparations it was unaffected by 10 mM galactonolactone, whereas in lucerne and limpet preparations there was less than 50 % inhibition.

β-Glucuronidase from mouse liver or limpet was unaffected by any compound other than saccharo-1:4-lactone and boiled mucate solution. Inhibition by these was very much more powerful than anything observed with the other glycosidases and lactones. The relatively feeble action of saccharo-3:6-lactone on β-glucuronidase (Table 1) has been shown to result from intramolecular rearrangement to form the 1:4-lactone (Levvy, 1952). It is interesting to note that the inhibitory power of mucate was not diminished after repeated recrystallization of the impure commercial product: the figures in Table 1 are very similar to those obtained previously (Levvy, 1952).

With the α- and β-mannosidases and glucosidases from whatever source, inhibition by the lactones was fairly selective inasmuch as a pronounced effect was seen only with the lactone of the correct configuration. In this group of enzymes, however, some small, but significant, inhibitory effects were noted with arabonolactone (cf. Jermyn, 1955). The arrangement of the secondary hydroxyl groups in this lactone is identical with that at carbon atoms 3, 4 and 5 in a mannoside or glucoside. β-Glucosaminidase, where the same argument applies, was slightly inhibited by arabonolactone, but by no other lactone studied (Table 2). Both gluconolactones caused slight inhibition of α-mannosidase from lucerne seed, but not from the limpet, whereas limpet β-glucosidase was feebly inhibited by both galactono- and xylono-lactone.

The most difficult results to interpret are those for β-galactosidase and β-xylosidase. Limpet β-xylosidase appears to be identical with the β-glucosidase in the preparation: both enzyme activities were equally affected by the gluconolactones. The affinity of this unspecific enzyme for both substrate and inhibitor seems to be less for xylose than for glucose derivatives, since xylonolactone, a powerful inhibitor of the β-xylosidase activity, acted only feebly on the β-glucosidase activity, as already noted. In the rumen, however, β-xylosidase

activity, although partly a side-action of the β-glucosidase, appears to be mainly the property of a distinct enzyme, since the gluconolactones never caused more than small inhibition of the hydrolysis of the β-xyloside.

In the rumen, β-galactosidase appears to be an individual enzyme, since only galactonolactone caused any marked inhibition. In the limpet, however, the results for the hydrolysis of the β-galactoside present a very confused picture, for which it is difficult to find any simple and comprehensive explanation. Apart from powerful inhibition by galactonolactone, there was slight inhibition by xylonolactone and fairly strong inhibition by the gluconolactones. Inhibition by the gluconolactones could not be increased beyond a maximum of about 60% by increasing the inhibitor concentration (cf. Tables 2 and 3). This finding would appear to rule out the possibility that this enzyme, like β-xylosidase, is identical with β-glucosidase in the limpet. It would also appear to exclude the possibility that limpet β-galactosidase activity is due to a single enzyme, highly specific for the substrate, but less specific towards inhibition by the lactones.

Action of the tartaric acids on β-glucuronidase

The opportunity was taken in the course of this work to examine the effects of the isomeric tartaric acids on limpet and mouse-liver β-glucuronidase. Karunairatnam & Levvy (1949) suggested that this point might be worthy of investigation, but only L-tartaric acid was available to them, and this had no action on the mouse-liver enzyme in a concentration of 15 mM. Wong & Rossiter (1951) found that, in a concentration of 10 mM, L-tartaric acid caused 15% and *meso*-tartaric acid 60% inhibition of rabbit-blood β-glucuronidase. In the same concentration, L-tartaric acid was claimed by Mills *et al.* (1953) to produce 85% inhibition of an ox-spleen β-glucuronidase preparation.

In the present work, D- and L-tartaric acids, racemic acid and *meso*-tartaric acid were tested, in a final concentration of 10 mM, on β-glucuronidase of limpet and of mouse liver, with 0·625 mM phenolphthalein β-glucuronide as substrate. Only *meso*-tartaric acid had any inhibitory action on β-glucuronidase, producing a 45% inhibition of the enzyme from limpet and a 23% inhibition of the enzyme from mouse liver. Commercial racemic acid had some action, but this disappeared on purification. In case intermolecular rearrangement in a tartaric acid solution might lead to the production of an inhibitor for β-glucuronidase, the effect of boiling the solutions for 1 hr. at acid pH was examined, but this did not influence the results. The asymmetric carbon atoms in *meso*-tartaric acid, it should be noted, have the same configuration as carbon atoms 4 and 5 in glucuronic acid.

DISCUSSION

Earlier work with β-glucuronidase and β-glucosidase (Levvy, 1952; Conchie, 1954) suggested that any glycosidase should be powerfully inhibited by the aldonolactone with identical configuration of the secondary hydroxyl groups, irrespective of the size of the lactone ring. With the exception of α-galactosidase, this was true of the glycosidases studied in the present work.

Saccharo-1:4-lactone and boiled mucic acid solution were completely specific for β-glucuronidase amongst those glycosidases studied. These inhibitors and the substrate for the enzyme constitute, however, a special case in the present experiments, inasmuch as there is $-CO_2H$ instead of $-CH_2OH$ at carbon atom 6. It is noteworthy that inhibition by mucic acid is not due to an impurity: in the light of the results presented above, this raises the suspicion that, in spite of evidence to the contrary (Levvy & Marsh, 1952), β-glucuronidase may have some action on β-galacturonides. Preliminary results indicate that the newly discovered β-galacturonidase activity of limpet preparations (Conchie *et al.* 1956) is inhibited by boiled mucic acid solution.

Mannono-1:4-lactone was completely specific for α- and β-mannosidase. It should, however, be realized that the degree of specificity of an enzyme towards glycosides of different sugars may not always be paralleled by its response to the different aldonolactones. Some lack of specificity was noted in the inhibitory actions of the gluconolactones, as well as xylonolactone and galactonolactone, although the effects were pronounced (Table 3) only with the gluconolactones, and then only with the limpet-enzyme preparation. Some of the minor effects might, of course, disappear if the different enzymes were separated from each other. If, however, inhibition of a glycosidase by a lactone is any criterion of enzyme specificity, one must conclude that limpet β-glucosidase and β-xylosidase activity are due to one and the same enzyme, and that the β-galactosidase activity of the same preparation results from the actions of at least two distinct enzymes.

α-Galactosidase is not the only exception to glycosidase inhibition by the corresponding aldonolactone. Preliminary results for limpet α-glucuronidase and α-galacturonidase activity (Conchie *et al.* 1956) indicate absence of inhibition by saccharo-1:4-lactone and boiled mucic acid solution respectively. The failure of boiled mucic acid solution to inhibit α-galacturonidase activity was first noted by Dr W. W. Reid (private communication), using a pectinase preparation and α-di- and tri-galacturonic acids as substrates.

One possible application of the inhibition of glycosidases by lactones is illustrated by the work of

Dr G. N. Festenstein (private communication) in this laboratory, who found that the hydrolysis of cellobiose by extracts from mixed rumen micro-organisms was almost completely inhibited by glucono-1:4-lactone, whereas the hydrolysis of sucrose by a rumen holotrich Protozoa preparation (Oxford, 1955) was quite unaffected by the lactone. This confirms the belief that the protozoal invertase is a β-fructosidase and not an α-glucosidase.

Ezaki (1940) and Horikoshi (1942) employed gluconic and galactonic acids, as well as their lactones, as inhibitors, in an attempt to distinguish different types of β-glucosidase and β-galactosidase activity in Taka diastase and emulsin. It was not realized, however, that lactonization could account for the effects of the aldonic acids in enzyme assays done at a slightly acid pH. This factor may explain the apparent difference between the emulsin type of enzyme, assayed at pH 6 and unaffected by the aldonic acids, and the Taka diastase type, assayed at pH 5 and inhibited by the aldonic acids (cf. Levvy, 1952). This work has been extended to xylosidase by Morita (1953).

SUMMARY

1. With the exception of α-galactosidase, α- and β-glycosidases from rumen liquor, lucerne seed, limpet visceral hump and mouse liver were powerfully inhibited by aldonolactones of corresponding structure and configuration.

2. In many instances, the aldonolactones were completely specific in their actions, but in others some cross-effects were noted that could usually be attributed to a lack of complete specificity on the part of an enzyme.

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Oxidation of *iso*Citrate and Associated Phosphorylations

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No direct measurements have as yet been reported of the value of the phosphorylation quotient (P/O ratio) associated with the oxidation of *iso*citrate to oxalosuccinate. Ochoa (1947), studying the one-step oxidation of *iso*citrate to oxalosuccinate in the presence of arsenite, failed to obtain phosphorylation, probably owing to the uncoupling action of the inhibitor (Lehninger, 1949). Copenhaver & Lardy (1952) were unable to obtain the one-step oxidation of *iso*citrate in the presence of semicarbazide, but from experiments in which citrate was oxidized in the presence of malonate they calculated a P/O ratio of 3 for the reaction *iso*citrate → α-oxoglutarate.

P/O ratios based on measurements of the rate of incorporation of labelled phosphate into adenosine triphosphate (ATP) (Krebs, Ruffo, Johnson, Eggleston & Hems, 1953) were between 3·1 and 3·6 when citrate was the substrate, and correction for differential rate of exchange of the β phosphorus atom of ATP raised these values to between 3·4 and 4·2 (Whittam, Bartley & Weber, 1955).

This paper presents observations on the oxidation of *iso*citrate by rat-liver mitochondria under different experimental conditions.

EXPERIMENTAL

Tissue preparations. Rat-liver mitochondria were prepared by a modification of the method of Bartley & Davies (1954). The liver, after chilling in ice-cold 0·9% KCl, was homogenized for 45 sec. in 9 parts of 0·25 M sucrose with a stainless-steel homogenizer of the Potter–Elvehjem type. The homogenate was centrifuged at 300 *g* for 10 min. at 0° to remove nuclei and unbroken cells. This rate of centrifuging, which is slower than that commonly used, minimizes the sedimentation of the large mitochondria (Novikoff, Podber, Ryan & Noe, 1953). Microscopic examination of the supernatant from this centrifuging showed very few nuclei. Potassium chloride (11·2%, w/v) was added to the supernatant to give a final concentration of 0·9%. The mixture was centrifuged at 2000 *g* for 15 min. The sedimented mitochondria were twice suspended in 0·9% KCl and centrifuged for 7 min. at 2000 *g*. They were finally suspended in the required volume of 0·9% KCl.

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Special chemicals. Barium ATP (LePage, 1949) was converted into the sodium salt by treatment with Na_2SO_4. In this product the ratio ADP/ATP was about 0·15.

Carrier-free $KH_2{}^{32}PO_4$ was obtained from the Atomic Energy Research Establishment, Harwell. It was diluted with water (1 mc/10 ml.) and neutralized if necessary.

Sodium (+)-*iso*citrate was obtained by alkaline hydrolysis of dimethyl*iso*citric lactone (Krebs & Eggleston, 1944; Hogeboom & Schneider, 1950).

Triphosphopyridine nucleotide (TPN) of 80% purity (LePage & Mueller, 1949) and diphosphopyridine nucleotide (DPN) of 60% purity (Kornberg & Pricer, 1951) together with preparations of DPN–*iso*citric dehydrogenase (Kornberg & Pricer, 1951) and TPN–*iso*citric dehydrogenase (Grafflin & Ochoa, 1950) were gifts from Dr D. D. Davies.

Fumarase (Massey, 1952) was purified to the stage of the first $(NH_4)_2SO_4$ fractionation and potato apyrase was prepared according to Lee & Eiler (1951). Hexokinase (Berger, Slein, Colowick & Cori, 1946) was purified to stage 3.

All other chemicals were of analytical reagent grade.

Experimental procedures. Each Warburg vessel contained in the main compartment 2 ml. of particles suspended in 0·9% KCl, 0·2 ml. of substrate, 0·5 ml. of about 0·2 M sodium ATP, 0·2 ml. of 0·1 M potassium phosphate buffer, pH 7·4, and 0·2 ml. of 0·02 M-$MgCl_2$. The side arm contained 0·1 ml. of the diluted solution of $KH_2{}^{32}PO_4$ and the centre well 0·2 ml. of 2 N-NaOH with filter paper. The volume was brought to 4 ml. by the addition of water. The vessels were gassed with O_2 as described by Whittam *et al.* (1955). For anaerobic experiments a stick of freshly scraped yellow phosphorus was put in the centre well and the vessels were gassed with N_2.

All the incubations were carried out at about 20°. When *iso*citrate was the substrate the radioactive phosphate was usually tipped into the main compartment at the start of incubation. When the sample of ATP contained more than 20% of ADP the isotope was tipped in after incubation for 3 min., when the ratio ATP/ADP had reached a steady-state value.

Oxygen consumption was measured manometrically after an equilibration period of 10 min. As the rate of O_2 uptake with *iso*citrate or citrate as substrate remained fairly constant during the 10–30 min. incubation, it was possible to extrapolate to the zero time of incubation. After incubation, the vessels were placed in a dish of ice and water and the enzymic reactions stopped by the immediate addition of 0·5 ml. of 30% (w/v) trichloroacetic acid. After centrifuging, the trichloroacetic acid extracts were used for determination of phosphorus compounds and for other chemical estimations.

Table 1. *Oxygen uptake and incorporation of* ^{32}P *into ATP with varying amounts of substrate and mitochondria in the presence of* (+)-isocitrate

Each Warburg flask contained in the main compartment 2 ml. of rat-liver mitochondrial suspension in 0·9% (w/v) KCl, 0·5 ml. of 0·02 M sodium ATP, 0·2 ml. of 0·02 M-$MgCl_2$, 0·2 ml. of 0·1 M phosphate buffer (pH 7·4), 0·2 ml. of sodium (+)-*iso*citrate (0·1 M, 0·05 M or 0·04 M); in the side arm: 0·1 ml. of $KH_2{}^{32}PO_4$. Final vol. was brought up to 4 ml. with water. Gas phase O_2, temp. 20°. v was calculated by the method of Krebs *et al.* (1953) after 2 min. incubation.

Expt. no.	Final concn. of *iso*citrate (M)	Dry wt. of mitochondria added (mg.)	Q_{O_2} (μl. of O_2/mg. dry wt./hr.)	O_2 consumption (μg.atoms/ min.)	v (μg.atoms of P exchanged/ min.)	P/O	Ratio (after 20 min.):*
1	0·005	46	11	0·75	2·07	2·76	0·56
2–3	0·005	37	11·8	0·65	1·87	2·94	0·56
4	0·005	31	12·3	0·57	1·30	2·29	0·50
5	0·005	24	13·1	0·47	0·84	1·80	0·53
6	0·0025	61	12·8	1·17	2·02	1·73	0·72
7	0·0025	40	10·2	0·60	0·84	1·40	0·35
8	0·0025	12	2·8	.	.	.	0·23
9	0·002	60	12·5	1·12	2·52	2·25	0·77

* $\frac{\text{Specific radioactivity of ATP (counts/min./}\mu\text{g.atom of labile P)}}{\text{Specific radioactivity of inorganic P (counts/min./}\mu\text{g.atom of P)}}$

Chemical estimations. Citrate was estimated (Taylor, 1953) on the neutralized trichloroacetic acid extract after incubation with crude aconitase. The sum of citrate, *iso*citrate and *cis*-aconitate was calculated according to Krebs & Eggleston (1944).

*iso*Citrate was determined spectrophotometrically by TPN–*iso*citric dehydrogenase (Ochoa, 1948). The molecular extinction coefficient given by Horecker & Kornberg (1948) was used to calculate the amount of *iso*citrate present. Identification and estimation of α-oxoglutarate was carried out according to El Hawary & Thompson (1953). As α-oxoglutarate was the only keto acid found in the test solutions the phenylhydrazones were sometimes determined colorimetrically without prior chromatographic separation.

Phosphate esters were separated by paper chromatography with the formic acid–*iso*propyl ether solvent of Hanes & Isherwood (1949) or with the *iso*butyric acid–NH_3 solvent (Krebs & Hems, 1953) if ATP and ADP were to be separated. After wet-ashing of the chromatogram spots, the phosphorus was estimated according to Berenblum & Chain (1938) and Bartley (1953).

For measurement of radioactivity an M_6 liquid counter (Veall, 1948) was used. The results are corrected for isotope decay, dead time and background counts. The estimation of the radioactivity located in P^β and P^γ of ATP was made after action of apyrase at 0° according to Whittam *et al.* (1955).

Treatment of the data. When possible, P/O ratios were calculated as described by Whittam *et al.* (1955). In experiments where this was not technically possible or convenient, calculations were made by the formula of Stewart (1953) case 1, assuming that P^β and P^γ exchange at the same rate. The error in this case was minimized by calculating only for the short times of incubation.

RESULTS

Oxygen uptake and incorporation of ^{32}P *into ATP with varying amounts of* isocitrate *and mitochondria*

Table 1 shows the influence of substrate concentration and of the amount of tissue on the oxidation of *iso*citrate and on the P/O ratio as measured by the rate of incorporation of ^{32}P into ATP. The phosphorylation rate (v) fell more rapidly than the O_2 uptake with decreasing concentrations of mitochondria. For example, in Expts. 2 and 5 the dry weight fell from 37 to 24 mg. but v was halved and the O_2 uptake diminished by about 30%. Thus the efficiency of the oxidative phosphorylation was dependent on the amount of mitochondria. When the concentration of mitochondria was greater than

Table 2. *Comparison of the specific radioactivities of the* P^β *and* P^γ *atoms of ATP after incubation with* (+)-isocitrate (0·0025 M) *and rat-liver mitochondria* (40 *mg. dry wt.*)

Determination of radioactivity of P^β and P^γ of ATP was made after apyrase action according to Whittam *et al.* (1955). Trichloroacetic acid extract (1 ml.) was chromatographed in order to separate ATP from inorganic P (Krebs & Hems, 1953). After elution by 0·02 M-$NaHCO_3$, ATP was incubated for 80 min. at 0° with the following mixture: 0·1 ml. of 0·11 M-$CaCl_2$, 0·1 ml. of M sodium succinate buffer (pH 6·5), 0·1 ml. of potato apyrase and water to bring the total vol. to 1·5 ml. Enzyme action was stopped by adding 0·2 ml. of 30% trichloroacetic acid. After centrifuging, 150 μl. of supernatant was used for determination of ADP and orthophosphate. (Results refer to Expt. 7, Table 1).

Period of incubation after addition of radioactivity (min.)	Specific radioactivity of P atoms of ATP (counts/min./ μg.atom of P)		Ratio: $\frac{\text{specific activity of P}^\gamma}{\text{specific activity of P}^\beta}$
	P^β	P^γ	
0	0	336	.
2	524	4220	8·1
6	1364	6510	4·8
10	2170	7650	3·5
20	4240	9050	2·1
30	5260	8900	1·6

about 37 mg./4 ml. the P/O ratio became constant, at a concentration of *iso*citrate of 0·005 M.

The concentration of ATP was maintained in spite of variation in the O_2 uptake, amount of mitochondria present and the variable phosphorylation rate.

When the concentration of *iso*citrate was lowered a larger quantity of mitochondria was required to maintain the same rate of incorporation of orthophosphate into ATP (Table 1).

In some experiments the ratio specific radioactivity of P^β and P^γ atoms of ATP/specific radioactivity of orthophosphate phosphorus remained low even after prolonged incubation. For instance, in Table 1 (Expt. 7) this ratio was only 0·35 after a period of incubation of 20 min. To test whether this low incorporation was due to lack of exchange between P^γ and P^β atoms of ATP, the relative amounts of radioactivity in these atoms was measured in this experiment according to Whittam *et al.* (1955). Table 2 shows that the exchange between P^β and P^γ was normal. Thus it is only the exchange between P^γ and orthophosphate which was lowered.

Effect of fluoride on the oxidation of isocitrate *and on the rate of incorporation of* ^{32}P *into ATP*

When phosphorylation is measured by coupling the synthesis of ATP with the hexokinase system (Slater & Holton, 1953), it is necessary to add fluoride to inhibit adenosine triphosphatase (ATPase) activity. The interpretation of the results obtained in the presence of fluoride depends on the validity of the assumption that inhibition of ATPase is the only action of fluoride in this system and, in particular, that it has no effect on phosphorylation. This assumption has been tested by measuring the effect of fluoride on the rate of incorporation of radioactive phosphorus into ATP in the presence of *iso*citrate.

As shown in Table 3 fluoride does not inhibit this reaction. However, as fluoride can inhibit the oxidation of *iso*citrate (see below) similar experiments were made with succinate as substrate. No effect of fluoride was found. The P/O ratios obtained, 2·12 with NaF and 2·31 without NaF, agree with the published figures of Krebs *et al.* (1953) and Whittam *et al.* (1955).

Slater & Bonner (1952) have shown that succinic oxidase is inhibited by fluoride and that this inhibition is enhanced by the addition of phosphate. Similar observations were made on rat-liver mitochondria. There was a slight inhibition of succinate oxidation (5–10 %) in the presence of 0·04 M fluoride and 0·005 M phosphate. This inhibition increased to about 40 % when the phosphate concentration was raised to 0·025 M. Inhibition of the rate of oxidation did not affect the P/O ratio. When *iso*citrate was the substrate, fluoride inhibited the O_2 uptake at 0·0025 M *iso*citrate concn., but not at 0·005 M *iso*citrate concn. (see Tables 3 and 6).

Fluoride is known to be an inhibitor of myokinase (Kotelnikova, 1950; Barkulis & Lehninger, 1951; Slater, 1953). The isotopic equilibration of the P^β and P^γ atoms of ATP is probably accounted for by the action of myokinase (Whittam *et al.* 1955) and thus the rate at which the P^β and P^γ atoms of ATP exchanged would be expected to decrease in the presence of fluoride. This expected decrease is illustrated in Table 3 by the fall, in the presence of fluoride, of the value of the slope m_2.

In the mathematical treatment by Whittam *et al.* (1955), m_1 and m_2 are constants obtained from a logarithmic plot of the time course of uptake of radioactivity into ATP. The rate of exchange of the P^γ atom of ATP with orthophosphate is measured by m_1 and the rate of exchange of the P^γ and P^β atoms of ATP by m_2. In Table 3 (Expt. 2), m_2 in the presence of fluoride was zero and thus myokinase must have been virtually completely inhibited.

Table 3. *Influence of fluoride on oxygen uptake and on rate of incorporation of* ^{32}P *into ATP in rat-liver mitochondria in the presence of* isocitrate

Incubation in Warburg vessels at 20° in O_2. The incubation medium (4 ml.) contained 2 ml. of tissue suspension in 0·9 % KCl, 0·5 ml. of about 0·02 M sodium ATP, 0·2 ml. of 0·02 M-$MgCl_2$ and 0·2 ml. of 0·1 M phosphate buffer (pH 7·4). Solution (0·1 ml. of $KH_2{}^{32}PO_4$) in the side arm was tipped when the vessels were put into the bath. The centre well contained 0·2 ml. of 2 N-NaOH and filter paper. Sodium fluoride, when present: 0·04 M.

Expt. no.	Dry wt. of mitochondria (mg.)	Final concn. of *iso*citrate (M)	NaF	O_2 uptake (μg.atom/min.)	v* (Krebs *et al.* 1953)	P/O	m_2†	m_1†	v (Whittam *et al.* 1955)	P/O
1	46	0·005	–	0·75	2·07	2·76	−0·022	−0·482	2·55	3·4
	46	0·005	+	0·76	2·16	2·85	−0·0115	−0·406	2·52	3·3
2	61	0·0025	–	1·17	2·02	1·73	−0·035	−0·517	2·76	2·38
	61	0·0025	+	0·74	1·81	2·45	.	.	.	.

* v calculated after a period of incubation of 2 min. (μg.atoms of P exchanged/min.).

† m_1 and m_2 are constants used in calculating v (see Whittam *et al.* 1955). m_1 measures the rate at which the P^γ atom of ATP exchanges with orthophosphate and m_2 the rate at which the P^β and P^γ atoms of ATP exchange with each other.

Consumption of isocitrate *and the effect of fluoride thereon*

Table 4 shows the changes in amounts of the tricarboxylic acids when (+)-*iso*citrate was incubated with rat-liver mitochondria. As expected, the aconitase reaction rapidly removed *iso*citrate.

Table 4. *Consumption of* isocitrate *by rat-liver mitochondria*

*iso*Citrate was determined spectrophotometrically according to Ochoa (1947). Citrate was estimated (Taylor, 1953) on the neutralized trichloroacetic acid extract after incubation with crude aconitase. The sum of citrate, *iso*citrate and *cis*-aconitate was calculated according to Krebs & Eggleston (1944). (Results obtained from Expt. 7, Table 1.)

Period of incubation (min.)	Total tricarboxylic acids found (μmoles)	*iso*Citrate found (μmoles)	*cis*-Aconitate + citrate calculated (μmoles)
0	9·4	9·7	0
6	6·65	2·4	4·25
30	3·34	0·4	2·94

The relation between the O_2 uptake and the quantity of tricarboxylic acid consumed is given in Table 5. The ratio μg. atoms of O_2 used/μmoles of tricarboxylic acid used rose with time; even during the earliest period of incubation it was greater than 1 (Table 5), which indicates that the oxidation went beyond the stage of α-oxoglutarate.

When fluoride was added, the consumption of *iso*citrate was increased whilst the O_2 uptake remained the same or decreased. This reduced the above ratio in some cases to values less than 1 (Table 6, Expt. 1). This means that tricarboxylic acids have been removed by a non-oxidative reaction. The determination of α-oxoglutarate in these experiments conclusively showed that oxidation to α-oxoglutarate was not the only reaction, as the removal of 5·4 μmoles of tricarboxylic acids was accompanied by the production of only 1 μmole of α-oxoglutarate. The observation suggests an anaerobic removal of tricarboxylic acids, a point which will be discussed below.

Table 5. *Variation of the ratio oxygen uptake/tricarboxylic acids consumed with time of incubation and concentration of substrate*

The standard content of Warburg vessels has been given in Table 1.

Expt. no.	Dry wt. of tissue (mg.)	*iso*Citrate concentration in flask (M)	Time of incubation (min.)	Tricarboxylic acids used (μmoles)	O_2 used (μg.atoms)	Ratio: O_2 (μg.atoms) / tricarboxylic acid used (μmoles)
1	46	0·005	6	2·6	4·5	1·7
	46	.	30	8·2	22·5	2·7
4	31	0·005	20	8·3	11·4	1·4
5	24	0·005	30	10·0	14·1	1·4
6	61	0·0025	4	2·0	4·7	2·3
	61	.	10	2·6	11·7	4·5
	61	.	30	5·7	35·1	6·2
7	40	0·0025	6	2·75	3·6	1·3
	40	.	30	6·06	18·0	3·0
9	60	0·002	20	4·0	22·4	5·6
10	75	0·002	30	4·23	36·0	8·5

Table 6. *Influence of fluoride on consumption of* isocitrate *by rat-liver mitochondria*

Standard incubation conditions. Fluoride, when present: 0·04 M.

Expt. no.	Dry wt. of mitochondria (mg.)	Final concn. of *iso*citrate (M)	NaF	Period of incubation at 20° (min.)	O_2 uptake (μg.atoms)	Tricarboxylic acids used (μmoles)	Ratio: O_2 used (μg.atoms) / tricarboxylic acid used (μmoles)	α-Oxoglutarate formed (μmoles)
1	46	0·005	−	6	4·5	2·6	1·7	0·4
				30	22·5	8·2	2·7	.
	46	0·005	+	6	4·55	5·4	0·85	1·0
				30	22·8	8·3	2·7	.
2	61	0·0025	−	4	4·7	2·0	2·3	.
				10	11·7	2·6	4·5	.
				30	35·0	5·7	6·1	.
	61	0·0025	+	4	2·96	2·0	1·5	.
				10	7·4	4·4	1·7	.
				30	22·2	8·8	2·5	.

Oxidation of citrate in the presence of semicarbazide

Semicarbazide (0·04 M) was found to have no inhibitory action on the incorporation of ^{32}P into ATP after 20 min. when β-hydroxybutyrate was the substrate.

When citrate was the substrate, semicarbazide inhibited the incorporation of ^{32}P into ATP in liver homogenates or mitochondrial suspensions without affecting the O_2 uptake.

When fluoride and semicarbazide were added the value of v was increased, the O_2 uptake was slightly inhibited and the P/O ratio was about twice that observed with semicarbazide alone (0·73 instead of 0·33, Table 7, Expt. 4). The quantity of citrate removed was increased when fluoride as well as semicarbazide was added (13 μmoles instead of 10 μmoles after incubation for 60 min.). This is analogous to the observation that the removal of *iso*citrate is increased by fluoride. However, in all cases the ratio μg. atoms of O_2 used/μmoles of tricarboxylic acid used was still higher than 1 (Table 8), which indicates that semicarbazide was ineffective as a trapping agent for α-oxoglutarate.

In spite of the extremely low rate of incorporation of radioactivity into ATP the total amount of ATP did not diminish. Semicarbazide thus stabilized ATP, possibly by inhibition of ATPase activity. A test was therefore made of the effect of semicarbazide on the ATPase activity of the mitochondria under anaerobic conditions in the absence of substrate. Under these conditions only a slight (10–15 %) inhibition of the ATPase activity occurred on the addition of semicarbazide. This suggests that in the intact mitochondria, metabolizing in O_2, ATPase activity is in some way linked with the rate at which P is incorporated into ATP.

Rate of turnover of ATP under anaerobic conditions

Krebs *et al.* (1953) and Whittam *et al.* (1955) showed that anaerobic phosphorylation was low under the conditions used for the study of turnover of ^{32}P into ATP, being at most 10 % of that obtained aerobically. Table 9 shows anaerobic experiments in which the rate of turnover of ATP was determined in the presence of *iso*citrate. After 6 min. incubation at 20°, the concentration of ATP had fallen, particularly when 2:4-dinitrophenol (DNP) was present, but in all cases an appreciable incorporation of ^{32}P into ATP occurred. The incorporation was approximately halved by fluoride and DNP (Table 9, Expt. 1). In Expt. 2 the concentration of ATP remained constant during the time of incubation, which made it possible to calculate v. The average value obtained (0·78) was approximately one-third of that found under aerobic conditions with a similar amount of mitochondria.

Table 7. *Influence of fluoride on the oxidation of citrate in the presence of semicarbazide*

Besides the standard additions (see Table 1) each Warburg flask contained 0·02 M citrate and 0·04 M semicarbazide.

Expt. no.	Fluoride added (final concn.) (M)	Tissue preparation	Dry wt. (mg.)	O_2 uptake (μg.atoms/min.)	v (Krebs *et al.* 1953)	P/O	v (Whittam *et al.* 1955)	P/O	Ratio (after 30 min.)*	Citrate used (μmoles)
1	.	Whole homogenate	62	0·44	0·15	0·34	.	.	0·12	5 (30 min. incubation)
2	.	Whole homogenate	59	0·42	0·17	0·40	.	.	0·27	9 (80 min. incubation)
3	.	Mitochondria	58	0·67	0·22	0·33	.	.	0·20	10 (60 min. incubation)
4	0·04	Mitochondria	58	0·56	0·41	0·73	0·56	1·06	0·51	13 (60 min. incubation)

* $\frac{\text{Specific radioactivity of ATP (counts/min./μg.atom of labile P)}}{\text{Specific radioactivity of inorganic P (counts/min./μg.atom)}}$.

Table 8. *Citrate consumption by rat-liver mitochondria in the presence of semicarbazide*

Results from Expt. 2, Table 7. The vessels initially contained 80 μmoles of citrate and 0·04 M semicarbazide; other additions as in Table 1. Tricarboxylic acids were estimated after 60 min. incubation.

Fluoride added (final concn.) (M)	Total tricarboxylic acid found (μmoles)	Citrate found (μmoles)	*iso*Citrate found (μmoles)	*cis*-Aconitate calculated (μmoles)	Ratio: O_2 uptake (μg.atoms) / tricarboxylic acid removed (μmoles)
.	68·5	40·8	6·4	21·3	4·0
0·04	65·5	62·4	2·2	0·9	2·4

Table 9. *Rate of turnover of ATP under anaerobic conditions*

*iso*Citrate (final concn. 0·004 M) was incubated anaerobically with rat-liver mitochondria. The flasks were pre-incubated for 2 min. before tipping in $KH_2{}^{32}PO_4$; 46 mg. dry wt. of mitochondria was used in Expt. 1 and 40 mg. in Expt. 2. Otherwise, conditions were as in Table 3.

Expt. no.	Additions (final concn.) (M)	Time of incubation after addition of ^{32}P (min.)	ATP in vessel (μmoles)	v [μg.atoms of P exchanged/min. (Krebs *et al.* 1953)]	Ratio:*
1	.	0	5·7	.	0
	.	6	4·8	.	0·201
	Fluoride (0·04)	6	4·9	.	0·096
	DNP (0·0001)	6	1·3	.	0·078
2	.	0	9·4	.	.
	.	1·75	9·0	0·73	0·072
	.	3·25	9·2	0·86	0·138
	.	4·25	9·0	0·74	0·174
	Fluoride (0·04)	1·25	9·0	0·20	0·012

* Specific activity of ATP (counts/min./μg.atom of labile P) / Specific activity of inorganic P (counts/min./μg.atom of P).

Table 10. *Anaerobic phosphorylation when glucose and hexokinase was used as trapping agent*

Rat-liver mitochondria (43 mg. dry wt.) were incubated with *iso*citrate at 20°. The main compartment of the Warburg vessels contained: 2 ml. of mitochondrial suspension in 0·9% KCl or 2 ml. of 0·9% KCl, 0·1 ml. of 0·02 M sodium adenosine monophosphate, 0·1 ml. of 0·02 M sodium ADP, 0·2 ml. of 0·1 M phosphate buffer, 0·4 ml. of 0·4 M-NaF, 0·2 ml. of 0·02 M-$MgCl_2$ and 0·1 ml. of 0·15 M sodium (+)-*iso*citrate when added. The side arm contained 0·2 ml. of hexokinase solution (20 mg./ml.) and 0·1 ml. of 0·25 M glucose. The gas phase was N_2 (a stick of phosphorus was put in the centre well). Vessels were shaken for 2 min. at 20° before tipping in hexokinase + glucose. Incubation time, 6 min.

Addition	Glucose phosphate formed/vessel (μmole)
Mitochondria + *iso*citrate	0·38
Mitochondria	0·21
None	0·06

The incorporation of phosphate into ATP could be due to an exchange reaction accounted for by the reversibility of the system. This would not require energy (Boyer, Falcone & Harrison, 1954; Swanson, 1956). To check this point an experiment was carried out with the same preparation in which the hexokinase system served to trap the ATP formed (Table 10). This showed that some net synthesis occurred under anaerobic conditions, but at a rate no more than 4% of that calculated from the rate of incorporation. After allowing for the synthesis of glucose phosphate by the myokinase reaction from the added ADP and AMP there remains 0·17 μg.-atom of P fixed, compared with about 4·5 μg.atoms of P measured from the rate of incorporation. Thus the anaerobic incorporation was mainly due to an exchange reaction.

Effect of added DPN and TPN on the incorporation of phosphate into ATP in the presence of isocitrate

It is known that mitochondria transfer H_2 from *iso*citrate either to TPN (Grafflin & Ochoa, 1950) or to DPN (Plaut & Sung, 1954). The relative magnitude of the phosphorylation associated with the two pathways is not known.

To test this, DPN or TPN, and a DPN- or TPN-specific *iso*citric dehydrogenase were added to phosphorylating mitochondrial suspensions. Added DPN does not readily enter native mitochondria but does so after treatment of mitochondria with water (Lehninger, 1951). In a control experiment the effect of water treatment on the ability of the mitochondria to oxidize and phosphorylate was tested. One-half of a sedimented mitochondrial preparation was suspended in water and allowed to stand for

Table 11. *Effect of fluoride on the phosphorylation quotient for the oxidation of reduced DPN and reduced TPN*

Warburg flasks contained in the main compartment: 2 ml. of rat-liver mitochondria (dry wt. 42 mg.) in 0·9% KCl, 0·5 ml. of approx. 0·02M ATP, 0·2 ml. of phosphate buffer (pH 7·4), 0·2 ml. of 0·02M-$MgCl_2$, 0·1 ml. of 0·1M *iso*citrate, 0·1 ml. of 0·5M nicotinamide and either 0·2 ml. of 0·008M DPN solution and 0·2 ml. of DPN-specific *iso*citric dehydrogenase or 0·2 ml. of 0·002M TPN solution and 0·2 ml. of TPN-specific *iso*citric dehydrogenase. Water added to 4 ml. The side arm contained 0·1 ml. of $KH_2{}^{32}PO_4$. NaF, when present, 0·04M.

	O_2 uptake (μg.atoms/min.)	v [μg.atoms of P exchanged/min. (Krebs *et al.* 1953)]	P/O ratio
DPN + DPN-specific *iso*citric dehydrogenase	0·77	2·18	2·83
DPN + DPN-specific *iso*citric dehydrogenase + fluoride	0·21	0·56	2·68
TPN + TPN-specific *iso*citric dehydrogenase	1·00	2·36	2·36
TPN + TPN-specific *iso*citric dehydrogenase + fluoride	0·54	0·85	1·56

10 min. at 0°. Concentrated KCl solution was then added to bring the final concentration to 0·9%. The second half of the preparation was suspended directly in the same volume of 0·9% KCl solution. Water treatment increased the O_2 uptake from 0·64 to 1·02 μg.atoms/min. but somewhat diminished the phosphorylation quotient (2·92 instead of 3·08). Fluoride reduced the O_2 consumption in the presence of the DPN-specific *iso*citric dehydrogenase but did not affect the P/O ratio; in the presence of the TPN-specific enzyme both O_2 consumption and the P/O ratio were reduced by fluoride (Table 11). These results are in agreement with the assumption that the oxidation of reduced TPN is not as effectively coupled with phosphorylation as is the oxidation of reduced DPN. However, further work is necessary to assess the effect of fluoride on the phosphorylation associated with the oxidation of the two reduced coenzymes.

DISCUSSION

The main purpose of this investigation was to establish the phosphorylation quotient for the oxidation of *iso*citrate to α-oxoglutarate. On addition of *iso*citrate to liver mitochondria phosphorylation quotients of between 1·4 and 3·4 were observed (Tables 1 and 3). However, these values do not refer to conditions where the conversion of *iso*citrate into α-oxoglutarate was the only reaction. In most cases there was further oxidation of the α-oxoglutarate and even when the ratio (μg.atoms of oxygen uptake)/(μmoles of tricarboxylic acid used) was below 1 the amount of α-oxoglutarate found was only about one-fifth of that expected, which indicates a non-oxidative removal of tricarboxylic acid. This might be one of the two dissimilation reactions known to occur in bacteria (but not so far demonstrated in animal tissues), namely citrate → oxaloacetate + acetate (Wheat & Ajl, 1955), *iso*citrate → glyoxylate + succinate (Saz & Hillary, 1956), or a fission to oxaloacetate and acetyl–coenzyme A.

The calculation of the phosphorylation quotient from the rate of incorporation of ^{32}P into ATP was complicated by the fact that phosphate was incorporated into ATP anaerobically when *iso*citrate was present. The anaerobic rate was about 25% of the aerobic rate. This incorporation was essentially due to an exchange reaction not utilizing energy, as shown by the virtual absence of a net synthesis of ATP in the presence of the hexokinase system.

Under a variety of conditions, especially in the presence of semicarbazide and oxygen, the concentration of ATP was maintained, although little or no synthesis of ATP took place, as indicated by non-incorporation of ^{32}P (e.g. in Table 7). This can only mean that the ATPase activity was low under these conditions. Anaerobically, under otherwise identical conditions, ATP was hydrolysed relatively rapidly. Thus oxygen can, in the presence of semicarbazide, reduce the ATP-splitting activity of mitochondria. It follows that ATP can be maintained as a result of respiration in two ways, either by resynthesis from ADP and orthophosphate, or by inhibition of ATPase activity. When the second mechanism operates, low P/O ratios are found. This represents an 'uncoupling' of respiration from phosphorylation, which differs from that observed in the presence of nitrophenols in that it does not cause a net ATP breakdown.

It is evident that the ATPase activity of mitochondria is highly variable under aerobic conditions. It should be appreciated that 'ATPase' activity may be the result of several different kinds of enzyme systems. In the present context ATPase

activity is defined as any reaction which converts ATP into ADP. When the concentration of ATP remains constant the rate of this reaction can be measured by the rate of ^{32}P incorporation.

The rate of anaerobic incorporation of ^{32}P into ATP seems to depend on the nature of the substrate present. With *iso*citrate it was 25 % of the aerobic rate; with succinate, under comparable conditions, 3 % (Whittam *et al.* 1955) and with α-oxoglutarate 13 % of the aerobic rate (Krebs *et al.* 1953). Thus although the incorporation of ^{32}P into ATP may be expected to measure the rate of true ATP synthesis under some conditions, this is not necessarily correct for all conditions.

SUMMARY

1. Rat-liver mitochondria were incubated with *iso*citrate (and other substrates) under a variety of conditions. The rates of oxygen uptake, substrate removal and incorporation of ^{32}P into ATP were measured. Phosphorylation quotients between 1·4 and 3·4 were found when *iso*citrate was substrate.

2. Fluoride (0·04 M) inhibited the oxygen uptake in the presence of succinate (up to 40 %) or *iso*citrate (up to 30 %), but did not change the phosphorylation quotient.

3. Fluoride inhibited the exchange of P^{β} and P^{γ} atoms of adenosine triphosphate (ATP) presumably by inhibition of myokinase.

4. Fluoride reduced the ratio atoms of oxygen consumed/tricarboxylic acid consumed to values less than 1. The tricarboxylic acid removal was not accounted for by the appearance of an equivalent amount of α-oxoglutarate.

5. Semicarbazide (0·04 M) had no effect on phosphorylation when β-hydroxybutyrate was the substrate, but almost completely inhibited phosphorylation when citrate was the substrate. The oxygen uptake was not affected. In spite of non-phosphorylation the concentration of ATP was maintained. Anaerobically, semicarbazide did not appreciably inhibit adenosine triphosphatase activity.

6. Incorporation of ^{32}P into ATP occurred anaerobically when *iso*citrate was substrate, the rate being about 25 % of the aerobic rate. This rate is much higher than that observed in the presence of succinate or α-oxoglutarate. The anaerobic incorporation was lowered by fluoride and 2:4-dinitrophenol.

7. The P/O ratio associated with the activity of the triphosphopyridine nucleotide-specific *iso*citric dehydrogenase was 2·36; the ratio was reduced to 1·56 by fluoride (31 % inhibition). The P/O ratio associated with the activity of the diphosphopyridine nucleotide-specific *iso*citric dehydrogenase was 2·83; fluoride reduced this to 2·68 (6 % inhibition).

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Haem Pigments of Cytoplasmic Particles from Non-Photosynthetic Plant Tissues

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Keilin (1925) first described the cytochromes in a wide variety of whole tissues from animals and plants. Yakushiji (1935) and Okunuki (1939) later studied the distribution of several cytochromes in higher plants, and, in particular, in algae, from which Yakushiji (1935) extracted cytochrome *c*. Mann (1938) found that the haematin of non-photosynthetic plant tissues is largely concentrated in the meristematic zones. From the disrupted cotyledons of germinating *Phaseolus* seeds, Hill & Bhagvat (1939) and Bhagvat & Hill (1951) sedimented particles which contained a cytochrome system (cytochromes *a*, *b* and *c*) analogous to that found in heart muscle and yeast by Keilin (1925). Hill & Scarisbrick (1951) extended this work and described cytochrome *f*, which occurs only in chloroplasts. Another haem protein, called cytochrome b_3, was found by these workers in the press juice of macerated leaves and in non-photosynthetic tissues. Hill (1954) found cytochrome b_6 in chloroplasts from etiolated barley. Lundegårdh (1954*a*, *b*) has observed in bundles of wheat roots yet another pigment, cytochrome *dh*, which he considers to be identical with plant succinic dehydrogenase. Both Vernon & Kamen (1954) and Chance & Smith (1955) have described a number of haem pigments occurring in photosynthetic bacteria.

The haem proteins occurring in plant tissues are thus fairly well described in terms of their spectroscopic properties, but there is much less information concerning their localization and function in plant cells. Cytochrome *f* (Davenport, 1952; Davenport & Hill, 1952; Lundegårdh, 1954*a*) and cytochrome b_6 (Hill, 1954) both probably act as hydrogen acceptors in the photosynthetic pathway for oxidation of the reduced products formed during the photolysis of water by chloroplasts. The complex of cytochromes *a*, *b* and *c* observed by Bhagvat & Hill (1951) is typical of mitochondria and sarcosomes from animal tissues in which these haem pigments function as hydrogen acceptors during oxidation of succinic acid (see Chance, 1952; Keilin & Slater, 1953; Morton, 1955*a*). Martin & Morton (1955) found that microsomes isolated from beet petioles contained a pigment apparently identical with cytochrome b_3 of Hill & Scarisbrick (1951), and showed that this haem protein is associated with a cyanide-insensitive pathway for enzymic oxidation of reduced di- and tri-phosphopyridine nucleotides. In this paper, the term cytochrome b_3 (M. & M.) refers to the microsomal cytochrome of Martin & Morton (1955), and the term cytochrome b_3 (H. & S.) to the pigment observed by Hill & Scarisbrick (1951).

This paper reports further studies of the haem compounds of plants, particularly of the cytochromes associated with cytoplasmic particles. Whereas cytochrome *b* is associated with the mitochondria, cytochrome b_3 (M. & M.) is almost entirely localized in the microsomal particles. Cytochrome *b* is intimately associated with the succinic dehydrogenase system of plants. Cytochrome *dh* has not been detected in enzymically active mitochondria isolated from developing wheat roots and other plant tissues.

MATERIALS AND METHODS

Reduced diphosphopyridine nucleotide (*DPNH*). This was obtained from C. F. Boehringer and Soehne, Mannheim, Germany.

Sodium dithionite. Laboratory reagent grade $Na_2S_2O_4$ (British Drug Houses Ltd.) was used.

Ethylenediaminetetraacetic acid (*EDTA*). This was used as the disodium salt (Versene) obtained from British Drug Houses Ltd.

Pyridine. Analytical-grade reagent was redistilled over pellets of NaOH, only the initial distillate and a small coloured residue being rejected.

Nitrogen gas. Cylinders were supplied by British Oxygen Co. Ltd. as containing not more than 0·002% (v/v) of O_2.

Spectroscopy. For general qualitative investigations, a low-dispersion (Sorby–Browning type) microspectroscope was used. This instrument contains a prism that produces two incident light beams, thus permitting direct comparison of two spectra. This replaced the eyepiece of a standard laboratory microscope. For measurement of the wavelengths of absorption bands, the low-dispersion spectroscope was replaced by a Hartridge reversion type. The spectroscopes were calibrated against the 546 mμ. mercury line and frequently checked against a solution of reduced cytochrome *c* (α-band, 550 mμ.) from heart muscle. For quantitative estimation of the concentration of reduced haemochromogen, a Zeiss comparator microspectroscope was used.

Absorption spectrophotometry. A Beckman spectrophotometer (Model DU) was used. The wavelength scale between

226 and 656 mμ. was checked against known lines of the emission spectrum from a mercury-discharge lamp. In no case did the error exceed the tolerances given by the manufacturers (*Beckman Instruction Manual* 305, 1954, p. 8). Quartz cuvettes of either 0·5 or 1 cm. light path were used.

Reflectance spectrophotometry. The preparations, packed in crushed ice, were transported to Sydney, N.S.W., by air. Measurements were made at the National Standards Laboratory, C.S.I.R.O., Sydney, within 4–12 hr. after isolation of the particles. The paste was placed in a 0·25 cm. × 2·5 cm. diameter optical cell and the percentage reflectance transmittance determined between 400 and 700 mμ. with a General Electric self-recording Hardy-type spectrophotometer, and a band-width of about 10 mμ. A didymium standard was used for wavelength calibration.

Standard protein haemochromogen. This was prepared from a suspension of washed bovine red cells by heating to 60° with 0·1 N-NaOH, cooling and adding sufficient solid $Na_2S_2O_4$ to reduce the haemochromogen completely. For the pyridine haemochromogen, pyridine (25%, v/v) was added immediately before the $Na_2S_2O_4$. The concentrations of both solutions were estimated from the extinctions at 558 mμ., molecular extinction coefficients of $32{\cdot}5 \times 10^3$ being used in both cases.

Estimation of the concentration of protohaematin. The intensity of the broad α-band of the reduced pyridine protohaemochromogen of the preparation was compared visually with that of a suitable standard (see Elliott & Keilin, 1934; Hill & Hartree, 1953). The plant preparation was made alkaline with 0·1 N-NaOH, and pyridine (approx. 0·2 vol.) and a trace of $Na_2S_2O_4$ were added. The preparation was immediately transferred to a 1 cm. optical cell, which was sealed from air with a thin cover-slip. The intensity obtained with the standard preparation of reduced pyridine protohaemochromogen (see above) was then matched to that of the unknown by adjustment of the length of the light path, with a comparator microspectroscope.

Nitrogen. This was estimated by a micro-Kjeldahl procedure (Morton, 1955*b*).

Preparation of dispersions of cytoplasmic particles

The procedure used for the preparation of cytoplasmic particles varied slightly for each different tissue, being based on experience gained in the preparation of cytoplasmic fractions from beet petiole as described previously (Martin & Morton, 1956*a*). The choice of dispersion medium used previously (0·2 M sucrose in 0·2 M potassium phosphate buffer, pH 7·4) was largely determined by the requirement of minimum interference in chemical studies. These considerations did not apply to studies on the cytochrome constitution of the isolated fractions. The medium used contained sucrose (0·2 M), potassium phosphate buffer, pH 7·4 (0·1 M), potassium succinate (0·1 M), EDTA (0·01%, w/v) and cysteine hydrochloride (0·1%, w/v, added immediately before the disruption of the tissue). The medium was designed to keep the cytochromes largely in the reduced state in which they may be more stable (see Morton, 1955*c*), and to inhibit the activity of polyphenol oxidases. The tissue dispersions were prepared as described below. All operations after the collection of the tissue were carried out in a cold room at approx. 2°.

Silver-beet petiole. Young silver-beet plants were freshly harvested and the white petioles dissected free of leaf laminae and washed thoroughly with cold water. The petioles were chopped by hand into small pieces (approx. 0·5 cm. × 0·5 cm. × 0·1 cm.) and ground for about 2 min. with the medium (150 ml./100 g. of chopped tissue) in a large earthenware mortar previously cooled to about 0°.

Wheat roots. These were prepared from wheat germinated for about 48 hr., kindly supplied by Barrett's Food Company Pty. Ltd., Melbourne. The roots were removed by vigorous rubbing of the wheat on a coarse stainless-steel screen (8 holes/in.). Approx. 20 g. of material, consisting mostly of roots with some intermixed cotyledons (see Martin & Morton, 1956*b*), was obtained from each kg. of germinating wheat. About 100 g. of the tissue was then ground by hand in a pre-chilled mortar with 25 g. of washed sand and 200 ml. of dispersion medium.

Onion apical stem. From eight to ten germinating onion bulbs, the apical stem bases (approx. 25 g. of tissue) were dissected and chopped into small pieces. They were ground in a chilled mortar with 50 ml. of dispersion medium (as above but containing 0·2%, w/v, of cysteine hydrochloride).

'Arum' spadices. The flowers were rubbed from the spadices from five white 'arum lilies' (*Zantedeschia aethiopica*). Approx. 20 g. of the flowers was ground with 50 ml. of dispersion medium.

Fractionation of dispersions. After grinding, each dispersion was filtered through four thicknesses of muslin cheese cloth. Fractionation of these dispersions was carried out as described previously (Martin & Morton, 1956*a*).

In some cases during preliminary studies, the intermediate fraction was not separated. In most cases the sedimented particles were washed by suspending in 0·15 M-NaCl and recentrifuging as appropriate for the particular fraction (see Martin & Morton, 1956*a*). In this way the total material for any fraction was obtained in a single centrifuge cup, a distinct advantage for spectroscopic studies.

In addition to the above fractions, a 'total particle' preparation was sometimes isolated by centrifuging the tissue dispersion at 50000 *g* for 90 min.

EXPERIMENTAL AND RESULTS

Spectroscopic and spectrophotometric studies of whole plant tissues and isolated cytoplasmic particles

Whole tissues

Visual observations. Suitable thicknesses of the tissue were immersed for 15 min. in mM-KCN in potassium phosphate buffer (0·05 M), at pH 7·4, in order to inhibit the cytochrome oxidase activity. The absorption bands of the cytochromes so reduced were then observed with a microspectroscope. Petioles of silver beet (*Beta vulgaris*), apical stems of onion, spadices of 'arum lily' (*Z. aethiopica*) and bundles of wheat roots showed essentially the same absorption spectrum. A broad band was visible, extending from about 552 to 566 mμ., with a midpoint at about 556 mμ. This band could not be adequately resolved into its components. A weak band was usually visible at about 600 mμ., together with a third, even weaker band at about 530 mμ.

No absorption bands at 571 mμ. [or at 585 mμ. in the presence of KCN (Lundegårdh, 1954*b*)] were observed in wheat roots.

Fraction obtained at 1500 *g for* 15 *min.*

Visual observations. With some tissues, such as silver-beet petiole, an absorption band at 675 mμ. (attributed to chlorophyll) could be seen in this material. However, no other absorption bands could be detected.

Mitochondria

Visual observations. Mitochondria were examined as a firm pellet obtained after centrifuging. When reduced with $Na_2S_2O_4$, mitochondria from silver-beet petioles, onion apical stems and wheat roots showed essentially similar absorption spectra. Although the bands could be easily resolved with the low-dispersion microspectroscope, with the Hartridge reversion spectroscope only the edges of the bands could be properly measured. The strong absorption band in the green extended from 552–555 mμ. to 564 mμ. This is probably the fused α-bands of cytochromes of the *c* type (α-bands between 550 and 554 mμ.) and cytochrome *b* (α-band 562–564 mμ.). A band was also seen at 603–604 mμ. This is considered to be the composite α-band of cytochromes *a* and a_3.

When a few crystals of sodium succinate and a few drops of 0·01 M-KCN in potassium phosphate buffer (0·2 M, pH 7·4) were added to a pellet of freshly isolated mitochondria from wheat roots, the absorption bands of the several reduced cytochromes appeared. The spectrum was directly compared with that of cytochrome *c* from heart muscle with the low-dispersion spectroscope. Whereas the extreme edge of the α-band of the *c*-type cytochrome corresponded to the position of reduced heart-muscle cytochrome *c* (at 550 mμ.), the mean position of this α-band was estimated to be at about 552–554 mμ. The α-band of the *c*-type component could be quite well resolved and distinguished from the broader cytochrome *b* component (α-band at about 562 mμ.).

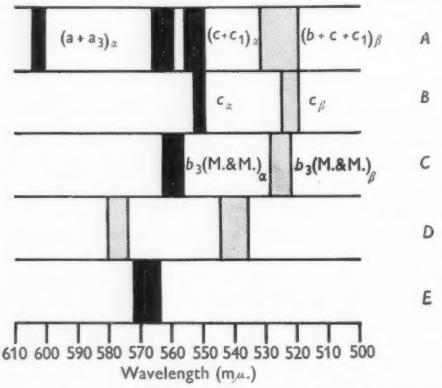

Fig. 1. Diagrammatic representation of visible absorption bands of plant haem pigments. *A*, Wheat-root mitochondria with sodium succinate and KCN; *B*, wheat-root mitochondria heated to 70°, with $Na_2S_2O_4$; *C*, wheat-root microsomes with DPNH (anaerobically), or with $Na_2S_2O_4$ (aerobically); *D*, precipitated supernatant proteins from silver-beet petiole, oxygenated; *E*, precipitated supernatant proteins from silver-beet petiole, with $Na_2S_2O_4$.

When the wheat-root mitochondria were heated at 70° for 30 min. and then reduced with $Na_2S_2O_4$, there appeared only a faint, narrow band, corresponding exactly with the position of the band of reduced cytochrome *c* from heart muscle. The same band appeared (together with a faint band at about 600 mμ.) when solid ascorbic acid was added to an unbuffered pellet of wheat-root mitochondria. Thus it appears that these mitochondria contain two cytochromes of the *c* type, cytochrome *c* with an α-band at 550 mμ., and considerably more cytochrome c_1 with an α-band at about 554 mμ. (see Discussion). The relative positions of the observed bands are shown diagrammatically in Fig. 1.

Addition of solid DPNH, together with buffered KCN (as above) caused appearance of bands at about 553 mμ., 562 mμ. (faint but definite) and at about 600 mμ., corresponding to the α-bands of cytochromes of the *c*, *b* and *a* types.

When a preparation of wheat-root mitochondria was reduced with $Na_2S_2O_4$, and KCN (about mM) was added subsequently to the pellet, there was no appreciable change in the absorption bands except for that at 604 mμ. This band appeared to shift toward the blue end of the spectrum. This was due to a widening of the band, so that the mean position of the broadened band was at 599 mμ. A similar widening of the band at 604 mμ. occurred after bubbling coal gas (used as a source of carbon monoxide) through a heavy suspension of wheat-root mitochondria. These observations suggest the presence of both cytochromes *a* and a_3 in wheat-root mitochondria.

A number of mitochondrial preparations from wheat roots have been examined as described above and also following treatment with liquid air as described by Keilin & Hartree (1949, 1955). No bands corresponding to those of the pigment called cytochrome *dh* by Lundegårdh (1954*b*) (α-band at about 571 mμ., shifting to about 585 mμ. with KCN) have so far been observed in the preparations reduced with either sodium succinate or $Na_2S_2O_4$.

Spectrophotometric studies. The absorption spectrum of the mitochondria from wheat roots was determined as described by Shibata, Benson & Calvin (1954) and by a method similar to that of Holton (1955) and Keilin & Hartree (1955). Some measurements were also made with clear dispersions

in sodium deoxycholate (cf. Strittmatter & Ball, 1952). However, most satisfactory results were obtained with a firmly packed pellet of the material placed so as to adhere to the side of a 0·5 cm. cuvette. The absorption of this pellet was read against a blank consisting of a mitochondrial or microsomal preparation in which the cytochromes had been destroyed by heating at 60° with 0·1 vol. of 30 % (w/v) H_2O_2. The concentration of this blank was adjusted so that an extinction of approx. 0·3 was obtained at 600 mμ. Fig. 2 shows the spectrum of mitochondria from beet petiole when reduced with $Na_2S_2O_4$. The maxima appear at 605, 558, 525 and 425 mμ.

Fig. 3 shows the reflectance spectrum of wheat-root mitochondria, both oxidized and reduced with $Na_2S_2O_4$. In the reduced preparation, maxima occur at 602, 556, 582 and 422 mμ. The difference spectrum (reduced minus oxidized) for this preparation is shown in Fig. 4. When plotted in this way, the maxima are seen at 603, 558, 526, 484 and 427 mμ. The difference spectrum (not shown) of wheat-root mitochondria, when oxidized and subsequently reduced with sodium succinate, has maxima at similar positions. The nature of the pigments responsible for these maxima is considered in the Discussion.

Intermediate fraction

On reduction with $Na_2S_2O_4$, this fraction invariably showed an absorption spectrum essentially the same as that of microsomes, except for an occasional weak band at about 605 mμ. This fraction was not systematically examined.

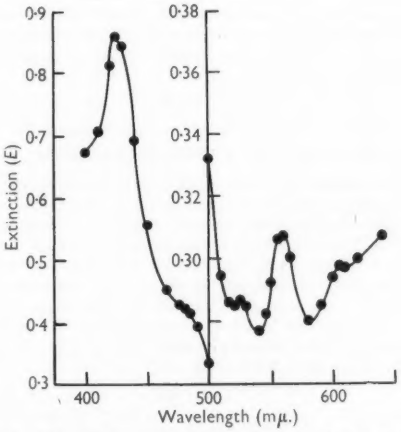

Fig. 2. Absorption spectrum of a firmly packed pellet of mitochondria from silver-beet petiole reduced with $Na_2S_2O_4$, and determined in the Beckman spectrophotometer as described in the text.

Microsomes

Spectrophotometric studies. Some of the properties of the cytochromes associated with plant microsomes have been described previously (Martin & Morton, 1955). The reduced pigment of beet-petiole microsomes had absorption maxima at 559·5, 525 and 425 mμ. The enzymic reduction of this cytochrome after addition of DPNH under anaerobic conditions, and the very rapid oxidation of the reduced cytochrome on exposure to air, were observed spectroscopically.

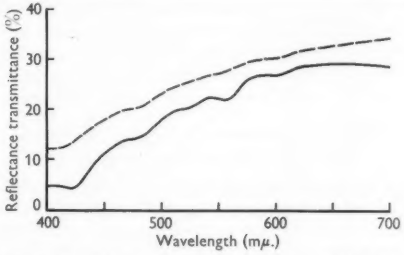

Fig. 3. Reflectance spectra of wheat-root mitochondria as determined with the General Electric self-recording spectrophotometer. Oxidized, – – –; reduced with $Na_2S_2O_4$, ——.

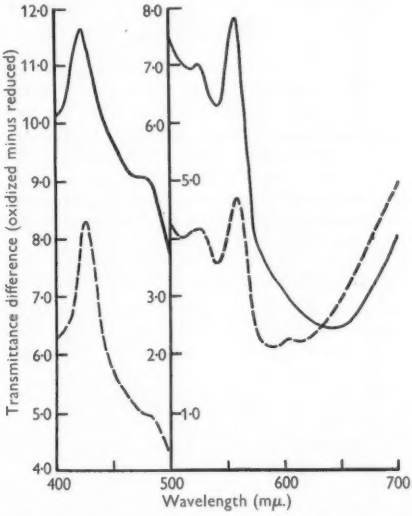

Fig. 4. Difference spectra (reduced with $Na_2S_2O_4$, minus oxidized) for mitochondria (– – –) and microsomes (——) from wheat root. Values were obtained by difference of the measurements obtained with the General Electric recording spectrophotometer as shown in Fig. 3 (mitochondria) and Fig. 6 (microsomes).

In further experiments, the anaerobic reduction of this cytochrome has been demonstrated spectrophotometrically as follows. A thick suspension of beet-petiole microsomes in 0·1 M potassium phosphate buffer, pH 7·4, was aerated thoroughly with oxygen and placed in a 1 cm. cuvette fused to the bottom of a Thunberg-type tube. The absorption spectrum between 500 and 600 mμ. of the oxidized microsomes was read against a blank treated with H_2O_2 (see above). The tube was then evacuated and the atmosphere replaced with oxygen-free nitrogen.

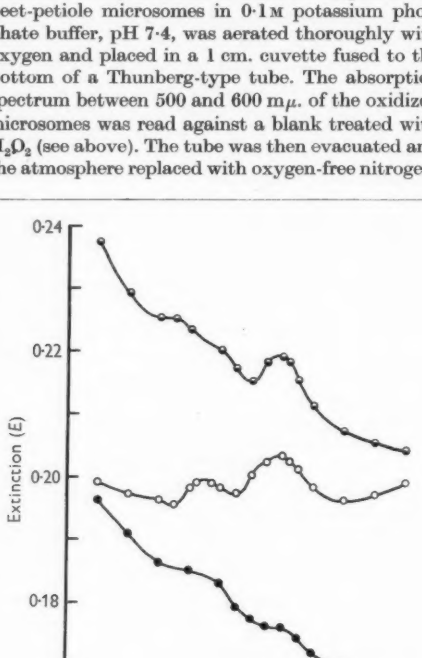

Fig. 5. Absorption spectra of a firmly packed pellet of microsomes from beet petiole, determined in the Beckman spectrophotometer as described in the text. Oxidized, ●; reduced with DPNH under anaerobic conditions, ◒; reduced with $Na_2S_2O_4$, ○.

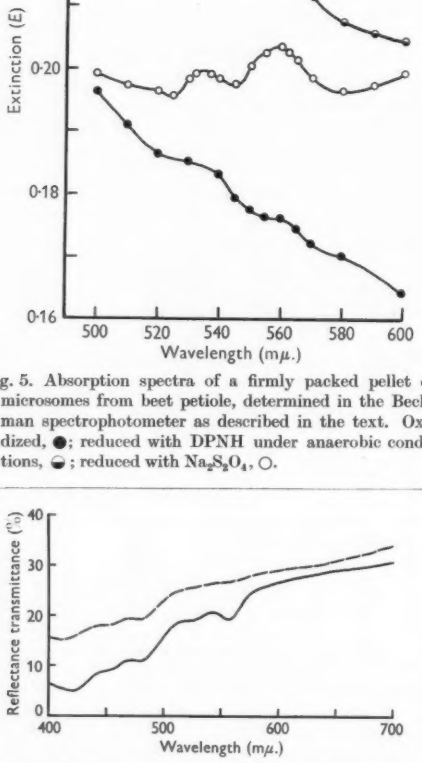

Fig. 6. Reflectance spectra of wheat-root microsomes as determined with the General Electric recording spectrophotometer. Oxidized, – – –; reduced with $Na_2S_2O_4$, ——.

The process was repeated three times. A small amount of solid DPNH from the side arm was tipped into the cuvette and mixed by agitation, and the absorption spectrum redetermined after incubation for 15 min. at room temperature. Finally, solid $Na_2S_2O_4$ was added to the cuvette and the absorption spectrum again determined. As shown in Fig. 5, the pigment was substantially reduced by DPNH under anaerobic conditions.

Fig. 6 shows the reflectance spectrum of a preparation of wheat-root microsomes, both oxidized, and reduced with $Na_2S_2O_4$. The maxima of the reduced preparation appear at 558, 526, 480 and 422 mμ. The difference spectrum for the preparation appears in Fig. 4, which shows maxima at 558, 525, 480 and 425 mμ. Measurements made with a firm pellet in the Beckman spectrophotometer as described for mitochondria gave maxima in the reduced preparation at 558, 525, 483 and 425 mμ., in substantial agreement with the results obtained with the General Electric instrument.

Localization of cytochrome b_3 (*M. & M.*) *in microsomes.* Cytochrome b_3 (M. & M.) has not been detected in either the precipitate obtained at 1500 *g* for 15 min. or in the supernatant fraction, but if present in the mitochondria it would be masked by the strong absorption bands of reduced cytochromes at 552–555 mμ. and at 563 mμ. Mitochondria and microsomes were therefore separately incubated anaerobically with sodium succinate (0·05 M) in the presence of 0·1 M potassium phosphate buffer, pH 7·4, and mM-KCN. After incubation at 37° for 30 min., the intensities of the absorption bands were compared with a protein haemochromogen preparation of known concentration. The preparations were then fully reduced with $Na_2S_2O_4$ and the cytochrome concentrations estimated as before. The result of one such experiment is shown in Table 1.

Table 1. *Estimation of haemochromogens in beet-petiole mitochondria and microsomes*

Mitochondria and microsomes were prepared by centrifuging dispersions of silver-beet petiole as described in the text. The haemochromogens were estimated after anaerobic incubation with 0·05 M sodium succinate at pH 7·4 in the presence of mM-KCN, and subsequently after reduction with $Na_2S_2O_4$. Measurements were made by comparing the intensity of the α-band of the reduced cytochromes with that of a standard solution of protein haemochromogen, prepared as described under Methods. Results are expressed as μg. of haematin/100 g. fresh wt.

	Haemochromogen formed when reduced with	
Cytoplasmic fraction	Sodium succinate	$Na_2S_2O_4$
10000 *g*, 15 min. (mitochondria)	3·00	3·00
50000 *g*, 90 min. (microsomes)	0	2·55

A preparation of microsomes from beet petiole was washed once with 0·15 M-NaCl and then assayed for DPNH–cytochrome *c* reductase activity as described previously (Martin & Morton, 1956*a*). It had an activity of 70 μmoles of cytochrome *c* reduced/hr./mg. of N at 20°. The haematin concentration in this preparation was estimated as $5{\cdot}3 \times 10^{-3}$ μmole of haematin/mg. of N (2·9 μg. of haematin/100 g. fresh wt.). From these figures, a value of 13000 μmoles of cytochrome *c* reduced/hr./μmole of cytochrome b_3 (M. & M.) haematin is obtained. In other preparations, slightly higher activities/μmole of haematin have been found (see Martin & Morton, 1955).

Final supernatant

Visual observations. In order to concentrate any haem pigments in the soluble fraction remaining after centrifuging a beet-petiole dispersion at 50000 ***g*** for 90 min., the supernatant was dialysed (in Visking cellulose tubing) against saturated ammonium sulphate at 0° for 18 hr. The precipitate was collected by centrifuging at about 2000 ***g*** for 20 min. at 0° and examined under the low-dispersion microspectroscope.

The preparation showed no distinct absorption bands. Several different portions of the wet precipitate were then treated as follows.

When $Na_2S_2O_4$ was added, an intense broad band appeared, extending from 565 to 570 mμ., with a mid-point at about 568 mμ. No other absorption band was observed (see Fig. 1). When coal gas (used as a source of carbon monoxide) was bubbled through this preparation (reduced with $Na_2S_2O_4$), the absorption band was shifted by about 4 mμ. toward the red end of the spectrum, and a weak band appeared at about 536 mμ. Addition of KCN (mM) to another portion of precipitate (reduced with $Na_2S_2O_4$) also appeared to shift the absorption band somewhat toward a longer wavelength.

The reduced pyridine haemochromogen formed with another portion of the precipitate showed very intense bands at 557 and 530 mμ., whereas the reduced imidazole haemochromogen showed bands at 562 and 530 mμ.

When oxygen was bubbled through another portion of precipitate, faint bands appeared at about 578 and 540 mμ. (see Fig. 1). These bands slowly disappeared on standing. No absorption bands could be observed after the subsequent addition of potassium ferricyanide at pH 5·0. No pigment could be detected in an ether extract of a portion of the precipitate. However, when a further portion was treated with 0·5 N-HCl for 2 hr. at room temperature, and the ether extract evaporated to dryness, a pigment was obtained which formed a reduced pyridine haemochromogen similar to that formed from protohaemin.

Distribution of haem compounds among the cytoplasmic fractions from beet petiole

Table 2 shows the distribution of haem compounds among the cytoplasmic fractions from beet petiole. The 'total dispersion' figures were obtained by adding the results for a 'total particle' preparation to those for the precipitate obtained on dialysis of the supernatant fraction (in Visking cellulose tubing) against saturated ammonium sulphate. Only 84 % of the total haematin of the dispersion was recovered.

DISCUSSION

The special difficulties associated with spectrophotometry of turbid materials, such as suspensions of cytoplasmic particles, have recently been investigated by several workers (see Shibata *et al.* 1954; Holton, 1955; Keilin & Hartree, 1955). The difference spectra (reduced minus oxidized compounds) are particularly valuable for differentiating compounds which may not be adequately resolved

Table 2. *Distribution of haem compounds in cytoplasmic particles from silver-beet petiole*

Particulate fractions were prepared from silver-beet petiole dispersions (see text). The haem compounds were estimated by forming the pyridine haemochromogen as described under Methods. Analyses for the total dispersion were obtained by adding the results for a 'total particle' preparation (see text) to those for the supernatant. Individual determinations are shown in parentheses below the mean values.

Cytoplasmic fraction	Haematin (μg./100 g. fresh wt.)	N (mg./100 g. fresh wt.)	Haematin (μmoles/g. of N)	Percentage of total dispersion
Total dispersion	32·6	39·1	1·22	100
1500 ***g***, 15 min. (debris, nuclei)	0·7 (0·6, 0·7)	3·2 (3·3, 3·1)	0·28	2
10000 ***g***, 15 min. (mitochondria)	4·29 (4·22, 4·45, 4·20)	2·95 (3·0, 2·9)	2·14	13
50000 ***g***, 90 min. (microsomes)	2·59 (2·70, 2·55, 2·52)	2·45 (2·6, 2·3)	1·55	8
Supernatant	19·9 (19·8, 20·0)	30·5 (31·6, 29·3)	1·00	61
Recovery (%)	—	—	—	84

in the spectra of the reduced cytoplasmic particles. In the present investigation with the Beckman instrument, losses due to scattering of light by the particles have been reduced by using a thin layer of packed material. As an alternative approach, use has been made of reflectance spectrophotometry, theoretical aspects of which are discussed by Giovanelli (in preparation). Moss (1956) has used this method for quantitative assay of the cytochromes of bacteria. The method is quite sensitive, a paste of bacteria containing 1 μg. of protohaemin/g. dry wt. showing a change of 0·06 % reflectance transmittance at 560 mμ. (Moss, 1956).

In general, the results obtained with both the above procedures showed good agreement with visual observations made with a low-dispersion microspectroscope. Because of the great sensitivity of the latter method, especially when used with particles brought to the temperature of liquid air as described by Keilin & Hartree (1949, 1955), it has been used as the method of choice for establishing the presence (or absence) of any cytochrome component. Owing to the relatively large bandwidths of the commercial spectrophotometers, cytochromes with closely adjacent absorption peaks may not be adequately resolved with these instruments (see Keilin & Hartree, 1955).

Cytochromes of plant mitochondria

Cytochromes a *and* a_3. Bhagvat & Hill (1951) showed that particles (presumably mitochondria) sedimented from disrupted plant tissues contained cytochrome *a*. Plant mitochondria also show considerable cytochrome oxidase activity (Hill & Bhagvat, 1939; Bhagvat & Hill, 1951; Millerd & Bonner, 1953; Martin & Morton, 1956*a*). Since Keilin & Hartree (1939) have shown that cytochrome oxidase of animal tissues is probably identical with cytochrome a_3 of animal tissues, it would be expected that cytochrome a_3 would occur in plant mitochondria. The effects of potassium cyanide and carbon monoxide on the absorption band at 604 mμ. suggest that cytochrome a_3 occurs in wheat-root mitochondria. This is supported by Lundegårdh's (1951, 1952) spectrophotometric detection of the γ-band of an *a*-type cytochrome in bundles of wheat roots. Cytochrome *a*, in contrast to cytochrome a_3, has a relatively weak Soret band (Keilin & Hartree, 1939; Lemberg & Legge, 1949, p. 369), so that its detection in whole wheat roots would be unlikely. Lundegårdh (1952) also observed a 'shift' of the bands of the ferrocytochrome in the presence of either carbon monoxide or potassium cyanide. The failure to detect the Soret band of the *a*-type cytochromes in the absorption spectra of the mitochondria from wheat roots and from beet petioles is probably due to the comparatively low concentration of cytochrome a_3 in the plant mitochondria, and to the relative insensitivity of the spectrophotometric methods in this region of the spectrum. Clearly there is a need for a more detailed study of the cytochromes of the *a*-type of plant mitochondria.

Cytochromes b *and* dh. As shown in Figs. 2–4, the α-bands of the haem pigments of reduced mitochondria cannot be resolved spectrophotometrically (cf. also, for example, Holton, 1955). However, they may be readily differentiated with a low-dispersion spectroscope. The maxima shown in Figs. 2–4 are therefore considered to be the resultant of several different cytochromes, of which cytochrome *b* is predominant. The maxima of reduced cytochrome *b* of plant mitochondria probably occur at about 563, 529 and 427 mμ. Although cytochrome b_6 of Hill (1954) has similar spectroscopic properties, this pigment apparently occurs only in chloroplasts and it is improbable that it occurs also in plant mitochondria. Moreover, the cytochrome *b*-type pigment of wheat-root mitochondria is fairly readily denatured by acetone. This differentiates it from cytochrome b_6, which is more stable (Hill, 1954).

Lundegårdh (1954*b*) has described a pigment called 'cytochrome *dh*', observed in bundles of wheat roots. The difference spectrum of this cytochrome shows a maximum at about 571 mμ., shifting to about 585 mμ. in the presence of potassium cyanide. If the pigment is identical with succinic dehydrogenase, as suggested by Lundegårdh (1954*b*), then it should be found in wheat-root mitochondria, which have a high succinic dehydrogenase activity (Martin & Morton, 1956*b*). It has been shown that succinic dehydrogenase is localized in the mitochondria in other plant tissues (Martin & Morton, 1956*a*). However, no such pigment has yet been observed spectroscopically either in the mitochondria or in other fractions isolated from developing wheat roots. Improvement of the sensitivity by freezing the preparations at the temperature of liquid air (Keilin & Hartree, 1949, 1955) has not enabled detection of this pigment. Lundegårdh (1954*a*, *b*) found that cytochrome *dh* occurred at about the same concentration as cytochrome *b* in whole wheat roots. The spectrum of wheat-root mitochondria fails to show any pigment corresponding to cytochrome *dh*, although cytochrome *b* is very prominent (Figs. 2 and 3). A possible explanation of this discrepancy is that very young (about 48-hr.-old) roots were used in the present investigation, whereas apparently quite mature roots (from plants 2–3 weeks old) have been used in Lundegårdh's studies (see Lundegårdh, 1951). Unfortunately, the exact nature of the material used is not stated in the later paper (Lundegårdh, 1954*b*). It is of interest that Lundegårdh (1952) observed the pigment with an

absorption maximum at about 482 mμ. which is present in wheat-root mitochondria (Figs. 3, 4) and wheat-root microsomes (Figs. 4, 6) as well as in beet-petiole microsomes (Martin & Morton, 1955).

Cytochrome *b* of plant mitochondria appears to bear a relationship to the plant succinic dehydrogenase system similar to that which cytochrome *b* of heart muscle bears to the succinic dehydrogenase system in this tissue. In the latter, cytochrome *b* apparently acts as a hydrogen carrier in the respiratory pathway and is quite distinct from succinic dehydrogenase itself. This enzyme, which was separated from cytochrome *b* by butanol treatment (Morton, 1950), has been shown to be a flavoprotein (see Morton, 1955*a*).

The succinic dehydrogenase system of plants appears to resemble closely that of animal tissues (Martin & Morton, 1956*a*), although it is somewhat more labile (Price & Thimann, 1951; Martin & Morton, 1956*a*, *b*). When mitochondria from beet or wheat roots are held at room temperature for several hours, the succinic dehydrogenase activity may decline very considerably without any apparent change in the intensity or position of the α-band of reduced cytochrome *b*. Thus it seems very likely that cytochrome *b* of plant mitochondria is quite distinct from succinic dehydrogenase, which is probably a flavoprotein as in animal tissues.

Cytochromes c *and* c_1. Goddard (1944) showed that cytochrome *c* isolated from wheat germ had essentially the same absorption spectrum and other properties as the similar pigment from heart muscle. Hill & Scarisbrick (1951) obtained cytochrome *c* (α-band, 550 mμ.) and cytochrome b_3 (H. & S.) (α-band, 559 mμ.) in solution in autolysates of several plant tissues. Cytochrome *c* has also been directly observed in wheat-root mitochondria (see Results), but in no other fraction from wheat roots. Cytochrome *c* was obtained in solution by autolysis of a beet-petiole dispersion (see Martin & Morton, 1955). There is therefore little doubt that all the plant tissues studied contain cytochrome *c*, which is entirely localized in the mitochondria.

However, when reduced with either sodium succinate or sodium dithionite, mitochondria from wheat roots and silver-beet petioles show a band at 554–555 mμ., rather than at 550 mμ. as might be expected. This band is unlikely to be due to cytochrome *f* (α-band, 555 mμ. when reduced), since this cytochrome has not been demonstrated to occur elsewhere than in chloroplasts of photosynthetic tissues (Davenport, 1952; Davenport & Hill, 1952). It seems more probable that this pigment is cytochrome c_1, first described by Yakushiji & Okunuki (1940) and recently shown by Keilin & Hartree (1955) to be identical with cytochrome *c* of Keilin & Hartree (1949). Cytochrome c_1 of heart muscle, when reduced, shows bands at 553, 524 and 418 mμ. (Keilin & Hartree, 1955; Estabrook, 1955). Keilin & Hartree (1955) found that the absorption maxima of the complex of cytochromes *c* and c_1 is at approx. 552 mμ. in heart muscle, in which the concentration of cytochrome *c* is in excess of that of cytochrome *b*. In plant mitochondria it seems likely that the absorption maximum of a mixture of cytochromes *c* and c_1 would occur at a longer wavelength, owing to the relatively low concentration of cytochrome *c* and the relatively high concentration of cytochrome *b*. Thus an absorption maximum of 553–554 mμ. could well be explained by the occurrence of cytochrome c_1 in the presence of cytochromes *b* and *c*. Visual observations (see Results) suggest that the concentration of cytochrome c_1 in wheat-root mitochondria is rather greater than that of cytochrome *c*.

Cytochrome of plant microsomes

The spectrophotometric studies described here (see Results and Figs. 4–6) confirm the earlier observations of Martin & Morton (1955) and extend them to microsomes from wheat roots. The possible function of cytochrome b_3 (M. & M.) in the DPNH–cytochrome *c* reductase and DPNH–oxidase activities of microsomes has been discussed previously (Martin & Morton, 1955, 1956*a*).

The evidence which suggests that the microsomal cytochrome is similar to a pigment observed by Hill & Scarisbrick (1951) has been published (Martin & Morton, 1955). However, as yet neither pigment has been extensively purified, so that their identity cannot be established beyond doubt. In order to avoid possible confusion, it now seems desirable to differentiate the pigments observed by these different authors in the manner suggested in this paper.

Neither the fraction obtained at 1500 *g* for 15 min. (containing plastids and nuclei) nor the final supernatant shows any absorption band in the 555–560 mμ. region when reduced with sodium dithionite. Whereas the cytochromes of mitochondria are completely reduced by anaerobic incubation with sodium succinate in the presence of potassium cyanide, cytochrome b_3 is not so reduced (Table 1). Hence plant mitochondria must contain negligible amounts of cytochrome b_3 (M. & M.), which appears to be entirely localized in the microsome fraction.

Pigments of the supernatant fraction

Table 2 shows that a considerable portion of the haem compounds of beet petiole remain in the supernatant fraction after centrifuging at 50 000 *g* for 90 min. The nature of these haem compounds is by no means clear. Catalase and peroxidase possibly comprise a small portion, as in other plant tissues (see Hill & Hartree, 1953; Lundegårdh, 1954*b*).

The appearance of bands at about 578 and 540 mμ. on oxygenation of the precipitate, followed by the slow disappearance of the bands (either under anaerobic conditions or in air), resembles somewhat the observations made on haemoglobins of legume-root nodules (Keilin & Wang, 1945), of protozoa (Keilin & Ryley, 1953), of baker's yeast (Keilin, 1953) and of some moulds (Keilin & Tissières, 1953). All these non-mammalian pigments form oxygenated ferrous complexes resembling mammalian oxyhaemoglobin. No ferric compound similar to 'acid' methaemoglobin was detected on oxidation of the precipitated proteins with potassium ferricyanide, but this may be due to the relatively low molecular extinction coefficient of the ferric compound (see Lemberg & Legge, 1949, p. 228). Keilin & Tissières (1953) and Keilin & Ryley (1953) likewise failed to detect any absorption bands after treatment of the mould and protozoan material with potassium ferricyanide. For the same reasons, the bands observed on oxygenation of the precipitated proteins are unlikely to be those of a ferric compound such as denatured protein parahaematin. The fading of the bands on cessation of oxygenation is probably due to the reducing activity of material (including traces of cysteine) in the precipitated proteins.

The compound which shows the two absorption bands on oxygenation of the precipitated proteins was observed in several different batches prepared as described, but the bands failed to appear when the proteins were precipitated at room temperature instead of at 0°. This suggests that the compound is rather labile.

The broad, strong absorption band which appeared at about 568 mμ. on addition of sodium dithionite to the precipitated proteins may be due to a denatured protein haemochromogen. This is indicated by the effects of added carbon monoxide and of added potassium cyanide, which probably form the carbon monoxide haemochromogen and the cyanide ferroporphyrin respectively. Denatured globin carbon monoxide haemochromogen shows bands at 571 and 545 mμ., and denatured globin cyanide ferroporphyrin at 568 and 540 mμ. (Lemberg & Legge, 1949, p. 228). These correspond rather closely with the positions of the observed bands (see Results).

It seems possible, therefore, that the extracts of beet petioles contain, in very small amount, a haem compound capable of forming an oxygenated ferrous complex. Most of the haematin of the supernatant fraction, however, appears to occur as a complex with denatured protein. This may be due to denaturation of haem compounds during the fractionation of the tissue dispersion and subsequent precipitation with ammonium sulphate. If so, the compounds are particularly labile.

SUMMARY

1. The haem compounds of white petioles of silver beet, roots of germinating wheat, flowers from spadices of 'arum lilies' (*Zantedeschia aethiopica*) and apical stems of onion, have been studied. Pieces of tissue, tissue dispersions and fractions separated by differential centrifuging were used.

2. Low-dispersion microspectroscopes, a Beckman spectrophotometer and a General Electric self-recording Hardy-type spectrophotometer were used. The quantitative distribution of the haem compounds among the cytoplasmic particles of beet petiole has been determined.

3. The plant mitochondria contain cytochromes closely resembling in absorption spectra cytochromes *a*, *b*, *c* and c_1 of animal tissues. Cytochrome *b* is predominant. Cytochrome *dh* has not been detected in mitochondria from wheat roots or from silver-beet petioles. There is evidence that cytochrome a_3 occurs in wheat-root mitochondria.

4. Cytochromes *b*, *c* and c_1 of plant mitochondria are reduced enzymically on addition of sodium succinate, and on addition of reduced diphosphopyridine nucleotide. Cytochrome *b* appears to be intimately concerned in the succinic dehydrogenase system of plants. The nature of plant succinic dehydrogenase is briefly discussed.

5. A haemprotein, here called cytochrome b_3 (M. & M.), is localized almost entirely in the microsome fraction, and is reduced enzymically by the addition of reduced diphosphopyridine nucleotide.

6. Spectroscopic observations showed that the supernatant fraction obtained from beet petiole contained in very small amount a labile pigment capable of forming an oxygenated ferrous complex. However, most of the haematin in this fraction occurred as a complex with denatured protein.

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Levels of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Tumours

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It is probable that the availability of pyridine nucleotides as well as the relative proportions of the oxidized and reduced forms of these nucleotides are among the factors controlling the metabolic behaviour of cells. For this reason, previously published work (Glock & McLean, 1955*a*) on the distribution of oxidized and reduced diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) in animal tissues has now been extended to a variety of tumours. This supplements the recent investigation of Jedeikin & Weinhouse (1955), who determined the contents of oxidized and reduced diphosphopyridine nucleotide (DPN^+ and DPNH respectively) of several tumours by a spectrophotometric method employing alcohol dehydrogenase and appropriate substrates. Besides supplying additional data on the DPN^+ and DPNH contents of various tumours, the present investigation provides the first reliable figures for TPN contents. Oxidized and reduced pyridine nucleotides have been determined by extremely sensitive methods (Glock & McLean, 1955*b*) which involve coupling enzymically reduced pyridine nucleotides with their respective cytochrome *c* reductases and then following the rate of reduction of cytochrome *c* spectrophotometrically.

EXPERIMENTAL

Tumour material. Most of the animals bearing transplantable tumours were very kindly supplied by Professor A. Haddow of the Chester Beatty Research Institute, London. In addition, we are indebted to Dr R. Stein of the Marie Curie Hospital, London, for rats bearing Jensen sarcomas. Animals with liver tumours induced by *p*-dimethylaminoazobenzene were provided by this Institute. (For details of dietary procedure, see Campbell, 1955.)

Preparation of substrates and enzymes. These were prepared as described in a previous publication (Glock & McLean, 1955*b*) except that cytochrome *c* and D-glucose 6-phosphate, the latter in the form of the heptahydrate of the barium salt, were purchased from Sigma Chemical Co., St Louis. TPN–cytochrome *c* reductase was prepared from Löwenbraü (Munich) bottom-ale yeast.

Estimation of oxidized and reduced pyridine nucleotides. The oxidized and reduced pyridine nucleotides were determined respectively in neutralized acid and alkaline tissue extracts (Glock & McLean, 1955*b*). The reaction mixture for the TPN assay consisted of 0·5 ml. of 0·25 M glycylglycine, pH 8·7, 0·1 ml. of 0·05 M glucose 6-phosphate (sodium salt), 0·1 ml. (1 mg.) of glucose 6-phosphate dehydrogenase, 0·1–0·5 ml. of neutralized tissue extract and 0·1 ml. (0·25 mg.) of TPN–cytochrome *c* reductase in a total vol. of 2·4 ml. The reaction was started by the addition of 0·1 ml. of 0·001 M cytochrome *c* and the rate of reduction of cytochrome *c* was followed spectrophotometrically at 550 mμ. in 1 cm. cells against a blank containing only buffer, water and cytochrome *c*. DPN was estimated by a comparable procedure in which glucose 6-phosphate, glucose 6-phosphate dehydrogenase and TPN–cytochrome *c* reductase were replaced by 0·1 ml. of ethanol, 0·1 ml. (0·1 mg.) of alcohol dehydrogenase and 0·1 ml. of DPN–cytochrome *c* reductase. These assay procedures were standardized daily by using four levels of each nucleotide (0·1–0·8 μg. of DPN and 0·05–0·4 μg. of TPN). The DPN^+, DPNH, oxidized and reduced triphosphopyridine nucleotide (TPN^+ and TPNH respectively) contents of the tissue extracts were calculated from the increase in optical density at 550 mμ. between 1 and 6 min., after correction for enzyme and extract blanks.

RESULTS

Results for the DPN^+, DPNH, TPN^+ and TPNH contents of a variety of tumours and of a few normal tissues are given in Tables 1 and 2. Table 1 includes the values for the pyridine-nucleotide contents of normal rat liver and also of liver tumours and liver from *p*-dimethylaminoazobenzene-treated animals. The liver tumours were in most cases mixed hepatomas and cholangiomas, but one pure hepatoma gave values that were not markedly different from the average for the mixed tumours. Although the total DPN ($DPN^+ + DPNH$) content of liver from tumour-bearing rats is not significantly different from that of normal liver, the average value for liver tumours is only about one-half of this. The total TPN (chiefly TPNH) contents of the same pieces of tissue show much more striking differences. Even in the liver from tumour-bearing rats the total TPN content is less than one-half that of normal liver, whereas the average value for the liver tumours is as low as 14 % of the value for normal liver.

Table 1. *Pyridine-nucleotide contents of normal livers and of liver tumours from* p-*dimethylaminoazobenzene-treated rats*

The assay conditions are given in the Experimental section. Figures in parentheses represent no. of animals. Results are expressed as means ± S.E.M.

Tissue	Pyridine-nucleotide contents (μg./g. of tissue)					
	DPN^+	DPNH	$DPN^+ + DPNH$	TPN^+	TPNH	$TPN^+ + TPNH$
Normal rat liver (23)	398±10	182±8	580±16	7±1	217±5	224±5
Liver tumours:						
Mixed cholangiomas and hepatomas (7)	226±15	83±12	309±19	3±2	27±9	30±9
Hepatomas (1)	255	25	280	<3	28	28
Liver from tumour-bearing rats (5)	441±75	151±35	592±32	4±1	92±21	96±23

Table 2. *Pyridine-nucleotide contents of transplantable rat and mouse tumours*

The assay conditions are given in the Experimental section. Figures in parentheses represent no. of animals. Results are expressed as means ± S.E.M.

Tissue	Pyridine-nucleotide contents (μg./g. of tissue)					
	DPN^+	DPNH	$DPN^+ + DPNH$	TPN^+	TPNH	$TPN^+ + TPNH$
Rat skeletal muscle (5)	278±16	27±5	305±19	8±1	9±2	17±2
Mouse skeletal muscle (4)	314±18	22±4	336±20	<3	<3	<3
Mouse thymus (4)	165±30	65±6	230±27	<3	9±1	9±1
Jensen rat sarcoma (5)	118±12	41±9	159±21	<3	4±1	4±1
Crocker mouse sarcoma (5)	112±22	61±6	174±27	5±2	9±3	14±4
Sarcoma 37 (5)	111±17	22±3	133±17	<3	<3	<3
Carr lymphosarcoma (4)	134±7	37±7	171±9	<3	<3	<3
EL4 mouse leukaemia (5)	101±7	33±2	134±7	<3	<3	<3
Walker rat carcinoma (5)	94±9	50±13	145±24	<3	<3	<3
MC/63 mouse carcinoma (5)	131±31	40±3	172±30	<3	<3	<3
Krebs ascites tumour (4)	132±13	36±0·3	168±13	<3	<3	<3

Results for the pyridine-nucleotide content of various transplantable solid tumours and for skeletal muscle and lymphatic tissue (thymus) are given in Table 2. The total TPN contents of all the tumours are extremely low or negligible. The total DPN contents of these transplantable tumours are, in general, lower than those found in most normal tissues (Glock & McLean, 1955*a*), although they do not differ markedly from such tissues as thymus, lung, testis and thyroid. In contrast with the wide range in total DPN contents of normal tissues (100–600 μg./g. of tissue), these solid tumours show a much narrower range (134–174 μg. of total DPN/g. of tissue).

The relative proportions of the oxidized and reduced forms of both pyridine nucleotides are not significantly different in normal and neoplastic tissues. In both types of tissue DPN is present preponderantly in the oxidized form and TPN largely and often almost exclusively in the reduced form.

DISCUSSION

In a recent valuable review by Weinhouse (1955) certain abnormalities in the oxidative reactions of tumour homogenates and mitochondria are discussed in terms of availability of pyridine nucleotides. Tumour homogenates and mitochondria have been shown to be much more dependent on an additional supply of DPN than are similar preparations from normal tissues in order to oxidize pyruvate and components of the citric acid cycle (Wenner, Spirtes & Wienhouse, 1951; Wenner & Weinhouse, 1953; Williams-Ashman & Lehninger, 1951; Kennedy & Williams-Ashman, 1952; Williams-Ashman & Kennedy, 1952). This pronounced DPN requirement of tumour mitochondria has not yet been satisfactorily explained. It could be due to a low DPN content of intact tumour cells, to a loose conjugation of the nucleotide with mitochondrial apoenzymes together with increased mitochondrial permeability, to a high diphosphopyridine nucleotidase activity, or to an impaired ability of tumour cells to synthesize pyridine nucleotides.

The results of Jedeikin & Weinhouse (1955) on the DPN^+ and DPNH contents of a number of normal and neoplastic mouse and rat tissues indicate a generally much lower content of total pyridine nucleotide in neoplastic tissues. In both types of tissue, however, the same pattern of oxidized and reduced forms was observed, the oxidized form being always preponderant. A low DPN content of tumours had also been observed by earlier workers (Bernheim & Felsovanyi, 1940; Kensler, Sugiura & Rhoads, 1940; Fisher & Schlenk, 1948; Carruthers & Suntzeff, 1953), but most of these results are open to criticism on account of the non-specificity of the methods used; in some cases only total pyridine nucleotide was determined. This low total DPN content of tumours cannot be due to increased destruction of this nucleotide since the work of Quastel & Zatman (1953) shows that the diphosphopyridine nucleotidase activity of a variety of tumours is within the range of normal tissue activities. Recent work of Branster & Morton (1956) indicates that nuclear DPN pyrophosphorylase activity is significantly reduced in mammary-gland carcinomata from C_3H mice. Whether this reduced capacity for DPN synthesis is a general property of all neoplastic tissue is at present unknown, although Waravdekar, Powers & Leiter (1956) have published a preliminary note to the effect that DPN synthesis in liver homogenates of CAF mice bearing sarcoma 37 is considerably reduced.

In the present investigation the TPN^+ and TPNH contents of tumours have been determined in addition to DPN^+ and DPNH. These figures for TPN appear to be the first reliable ones to be published. Our values for DPN^+ and DPNH as well as for the relative proportions of the oxidized and reduced forms are in very good agreement with the recent results of Jedeikin & Weinhouse (1955) and support the general view that the total DPN content of tumours is relatively low. Of great interest is the finding that the total TPN (chiefly TPNH) contents of all the tumours investigated are extremely low or even negligible. The gradual reduction in the total TPN content of rat liver undergoing carcinogenesis by *p*-dimethylaminoazobenzene is most striking. The average values for normal rat liver and liver and liver tumours from tumour-bearing rats are respectively 224, 96 and 22 μg. of total TPN/g. of tissue. Much less dramatic changes were found in the total DPN contents of the same pieces of tissue, in contrast to earlier findings of Kensler *et al.* (1940). These results suggest, in addition, that the synthesis of TPN from DPN is severely curtailed in these tumours. The relative proportions of oxidized and reduced forms of both pyridine nucleotides are not significantly different from those of normal tissues (Glock & McLean, 1955*a*). In both neoplastic and normal tissues DPN is present preponderantly in the oxidized form and TPN largely and often almost exclusively in the reduced form. The finding of Euler, Schlenk, Heiwinkel & Högberg (1938) that Jensen sarcomas contain much more DPNH than DPN^+ has not been confirmed.

Weinhouse (1955) has re-emphasized the very marked dependence of glycolysis on the availability of various cofactors, and has suggested that the characteristically high rate of glycolysis of tumours might conceivably be due to an abnormal intracellular distribution of necessary cofactors,

including pyridine nucleotides. Carruthers & Suntzeff (1954) and Carruthers, Suntzeff & Harris (1954) have studied the distribution of total pyridine nucleotide in cellular fractions of normal tissues, various transplantable tumours and *p*-dimethylaminoazobenzene-induced cholangiomas. Although the total pyridine nucleotide content of many of these tumours was low, the pattern of distribution among the various cellular fractions was not markedly different from that of normal tissues, although very little, if any, pyridine nucleotide could be detected in the microsomal fraction. We have determined the intracellular distribution of DPN^+, DPNH, TPN^+ and TPNH in normal rat liver (Glock & McLean, 1956) and a few preliminary experiments have indicated that both total DPN and particularly total TPN contents of mitochondria are very low in *p*-dimethylaminoazobenzene-induced liver tumours. This work is being continued.

Previous work on the pyridine-nucleotide content of normal tissues (Glock & McLean, 1955*a*) indicated that the total TPN contents showed a rough positive correlation with levels of activity of the TPN-dependent 6-phosphogluconate oxidative pathway. This correlation does not seem to apply to tumours, however, since glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities of a variety of tumours were found to fall within the range of normal tissues (Glock & McLean, 1954), although all the tumours used in the present investigation had very low TPN contents. Additional evidence indicating an active 6-phosphogluconate pathway in neoplastic tissues is provided by experiments using [1-^{14}C]glucose and [6-^{14}C]glucose (Agranoff, Brady & Colodzin, 1954; Abraham, Hill & Chaikoff, 1955; Emmelot, Bosch & van Vals, 1955; Kit, 1956).

SUMMARY

1. The oxidized and reduced forms of di- and triphosphopyridine nucleotide have been determined in a number of transplantable solid tumours and also in *p*-dimethylaminoazobenzene-induced liver tumours.

2. Although liver from tumour-bearing rats has a normal diphosphopyridine nucleotide (DPN) content, the total triphosphopyridine nucleotide (TPN) content is less than one-half that of normal liver. In liver tumours, however, the concentration of both pyridine nucleotides is considerably reduced, total TPN being affected much more than total DPN.

3. The total DPN contents of the solid transplantable tumours investigated are, in general, lower than those of most normal tissues. The total TPN contents are in all cases very low or negligible.

4. The relative proportions of the oxidized and reduced forms of both pyridine nucleotides are not significantly different in normal and neoplastic tissues.

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